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The publication of this supplement has been supported by an educational grant from Shire Pharmaceuticals

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Epidemiology of nonmelanoma skin cancer (NMSC) in Europe: accurate and comparable data are needed for effective public health monitoring and interventions

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Accepted for publication
5 March 2007

Key words
basal cell carcinoma, epidemiology, nonmelanoma skin cancer, squamous cell carcinoma

Conflicts of interest
C. Ulrich has acted as a lecturer for Shire Pharmaceuticals and E. Stockfleth has acted as a lecturer/consultant for Shire Pharmaceuticals. All remaining authors declare no conflict of interest.

Summary
Nonmelanoma skin cancer (NMSC) is the most common malignancy occurring in white populations. It is currently becoming an important challenge in terms of public health management as the increasing incidence rates will probably have a tremendous impact on healthcare costs. Possible factors driving this rise in NMSC numbers are increases in both acute and prolonged UV exposure together with increasing numbers of older people in the population. A better understanding of NMSC epidemiology in Europe is essential if an evidence-based European-wide public health policy is to be developed. It is obvious this can only be achieved by recording and analysing comparative epidemiological data. Finally, by improving the skin examination training for physicians, developing guidelines and exchanging best practices, a high level of healthcare could be provided for NMSC.

Nonmelanoma skin cancer (NMSC) is the most common cancer in Caucasian populations and is considered to be of epidemic proportions worldwide. Though the term NMSC covers all cutaneous cancers that do not involve melanocytes, it is widely (but perhaps inconveniently) used to refer to 2 major types of skin cancer: basal cell carcinoma (BCC) and squamous cell carcinoma (SCC). These two forms of skin cancer account for more than 95% of all NMSC.

Mortality from BCC and SCC is low but there may be substantial morbidity from disfigurement as the lesions tend to be located on the skin of the head and neck. As the prevalence of BCC and SCC is high they represent a considerable economic burden to national health systems. The exact number of people with BCC and SCC is unknown because in most countries these cancers are not reported in cancer registries. It is estimated, however, that more than one million cases of BCC and SCC occur in the United States every year. Furthermore the average annual increase of NMSC in white populations in Europe, the United States, Canada and Australia has been 3% to 8% since the 1960s, and although incidence rates are lower in countries further away from the equator, in every country where records exist, NMSC is on the increase. In a recent report from the Netherlands, it has been shown that the annual incidence of NMSC has increased rapidly over the past few decades. The increases were 2.4% in men and 3.9% in women for BCC and 1.2% in men and 3.4% in women for SCC.

In the absence of a global database there is a need to collect, process and analyse data about the incidence of NMSC across Europe for the purpose of effective public health monitoring. This is essential in order to obtain objective, reliable, compatible and comparable epidemiological information, which would enable both the decision makers and the healthcare professionals to formulate appropriate strategies, policies and actions to deal efficiently with this public health burden.

The importance of NMSC and Europe’s public health
Europe’s population is ageing. Although the population of the 27 Member States of the European Union (EU) is estimated to remain constant at around 450 million between 2000 and 2015, there will be a 22% increase in the number of people aged 65 and over, and a 50% increase in those aged 80 and over. Indeed, the number of people aged 80 and over is expected to nearly triple, rising from 18 million in 2004 to about 50 million in 2051. A consequence of the ageing of Europe’s
population will be a notable increase in the occurrence of all
cancers, 80% of cancers are diagnosed at 55 years and older and
skin cancer, although it is not unknown in children and young
adults, is essentially a disease of ageing populations.

The number of recorded cases of NMSC in the U.K. in
2001 was around 62 700.12 While it is generally accepted that
recorded numbers of these cancers underestimate the true fig-
ture, they serve to illustrate that 80% of cases occur in people aged 55 years and over. The incidence of BCC in South Wales in 1998 in individuals over 75 years old was approximately five times higher than for individuals aged between 50 and 55 years old. For SCC the figure was approximately 35 times higher.13 This is because the incidence of SCC increases more rapidly with age than BCC. For example, in Maastricht, the Netherlands, SCC was the most common form of cancer among the very elderly (aged over 95 years old).14

The incidence of any cancer has a great impact on the cost
of its management.4 The more frequent the cancer, the higher the annual cost of its management. Taking the above facts into account, and in the absence of effective intervention, NMSC can be clearly identified as one form of cancer that will become important in public health terms in the coming decades.5 Even if intervention starts now we will still have to find means of managing the part of the population that has already received unprotected or excessive sun exposure.

Cases of BCC and SCC, unlike other cancers, are often not tracked or even included in discussions or publications of cancer statistics. Importantly, there is no global European public health strategy for the management of NMSC. The most likely reasons for this are that BCC and SCC are associated with much less morbidity and mortality compared to other malignancies. They are, however, far more frequent than other cancers; NMSC incidence rates in Europe and there has been a series of publications giving insight into the epidemiology of BCC and SCC. Nearly half the lesions appeared on the face.3

In the EU over the past few decades, there has been an increase in health spending of almost 10% of Member States' gross domestic product (GDP).15,16 Considering the increase in life expectancy17 and the fact that the health sector constitutes an important part of the Member States' social and security systems, it is obvious that these systems will come under tremendous economic pressure due to increases in the cost of health services. It is tremendously important, therefore, for the EU to formulate appropriate policies and programmes on public health issues necessary to deal with these challenges.18

The financial impact of treatment, therefore, should also be to prioritise different malignancies in addition to classifying them by number of cases and number of deaths. Such an approach gives NMSC much more significance in the field of public health management than by simply relying on death statistics. Taking into account the already high and rising incidence, the cost of NMSC care to national health systems is likely to increase. Moreover, although the incidence of these tumours already seems very high, rates have probably been underestimated for a number of different reasons:22

1. Elderly patients tend to ignore lesions
2. Many registries do not collect data because these tumours are not specified on death certificates
3. Many cases are treated without histological analysis (for example with cryotherapy or topical agents), and therefore cannot be extracted from pathology files
4. Many registries, even important ones, group BCC and SCC under the term NMSC so that assessing definite incidence rates for these cancer types is very difficult
5. These tumours are frequent and therefore the information is difficult to collect. Moreover they are managed by different medical specialties and in different settings23,24
6. Recurrences and second NMSC lesions occur often and are mainly ignored by statistics and registries

Since the incidence of these lesions is underestimated it is likely that the workload they generate is also significantly underestimated by health economists and healthcare managers.25

There are no compatible and comparable data for NMSC in Europe

The incidence of both BCC and SCC is higher in fair skinned, sun-sensitive people.3,26 Europe’s population, according to a 2006 WHO report, consists of more than 98.38% lightly pigmented inhabitants (skin types I to IV).3 As already mentioned, the exact incidence of BCC and SCC is difficult to establish. On one hand there are not many cancer registries that record these cancers, while on the other some group BCC and SCC under the term of NMSC instead of collecting data separately.3 In addition, BCCs are frequently multiple and few if any cancer registries record secondary lesions once the first has been registered. Currently, no cancer registry exists that records data at an European level. However there are some registries that track incidence rates in Europe and there has been a series of publications giving insight into the epidemiology of BCC and SCC.

The ratio of BCC to SCC in white populations appears to lie between 4:1 (higher latitudes) and 2:5:1 (lower latitudes) with the incidence of SCC doubling for each 8–10 degree decline in latitude.24 There are no comparative studies examining whether this assumption is correct for Europe where latitude varies from between 30–40 degrees to 60–70 degrees.3 For the period 1993 to 1998 the Trentino Skin Cancer Registry in Italy calculated an incidence rate of 88 per 100 000 people for BCC and 29 per 100 000 for SCC.22 In Izmir, Turkey, SCC was equally common in men and women, whereas BCC was nearly three times more frequent in men.27 Nearly half the lesions appeared on the face.

In Vaud, Switzerland, BCC was the most common form of skin cancer reported in both sexes and the incidence has been rising steadily since registration was introduced in the mid-1970s.28 In Germany, between 1998 and 2001, the age-standardised rate for all forms of NMSC was 100.2 per 100 000 inhabitants per year for men, and 72.6 per 100 000 per year for women, with 80% of all tumours being BCC.29

Between 1978 and 1995, the Slovakian Cancer Registry registered 38,629 cases of NMSC; 19 600 in men and 19 029 in women. During this period, incidence rates for BCC
increased by 70.4% in men and 65% in women, while incidence rates of SCC increased by 13.5% in men and 18.8% in women. The head and neck were the most common sites (84.2% BCC and 74.7% SCC) followed by the trunk for BCC (17% in men and 11% in women) and upper limbs for SCC (12% in men and 12.5% in women).  

In Northern Ireland, the most commonly diagnosed NMSC reported was BCC, but no specific numbers were given: on average 1116 men and 1081 women were diagnosed as having NMSC each year. In Sweden, 39 805 SCCs were registered between 1961 and 1995. Incidence rates increased substantially in men (by 42%) and in women (by 146%) during this period, and interpretation of mathematical models led the authors to conclude that these increases could probably be explained by increased cumulative sun exposure and increasing incidence among the elderly. In Finland, the age-adjusted incidence rate of BCC between 1991 and 1995 was 49 per 100 000 person-years in men and 45 in women. For SCC, it was seven in men and four in women. Both cancer types showed an increasing trend in incidence rates.

The traditional incidence pattern, where BCC is more commonly seen in older patients, usually males, on areas of chronically exposed skin, such as the head and neck area, seems to be changing. A publication from the Netherlands, based on the Eindhoven cancer registry that records BCC separately, suggests that the ‘typical’ BCC patient in northwestern Europe is becoming younger, more often female, and is increasingly showing affected sites other than the head and neck area, i.e., more commonly the trunk. In this study, BCC incidence rates in women exhibited a cohort-effect, with still increasing rates in younger birth cohorts, implying a behavioural risk factor, probably sun exposure. The authors postulate that this behavioural risk factor would have an increasing influence over successive generations. As this effect is not discernable in men, it appears that the trend in men is mainly attributable to ‘drift’, possibly caused by intermittent overexposure to solar UV radiation, which increased more gradually over time in a broad age range.

Death from NMSC is not common but does occur. A recent WHO publication estimates a total number of 1017 deaths resulting from SCC and 672 from BCC for both sexes and all ages in the three WHO regions of Europe. Furthermore NMSC mortality in the EU presents an entirely different picture than that seen for melanoma. The rates are higher in men and women in southern European countries (Greece, Spain, Portugal and Italy) and low in the Nordic countries. Mortality from NMSC is almost always as a result of SCC that metastasises, and this accounts for up to 20% of all deaths from skin cancer.

An encouraging observation is that despite the increasing incidence of NMSC, mortality has been decreasing, probably due to early detection and excision of tumours before any metastasis has occurred. In Finland the mortality rate for BCC between 1991 and 1995 was 0.08 per 100 000 person-years in men and 0.05 in women, with a decreasing mortality trend. In the Netherlands, de Vries et al report a decrease of 1.9% in mortality of SCC over the past few decades.

Risk factors: what is driving the increasing incidence?

There are many reasons for the worldwide increase in NMSC incidence. Etiological factors that underlie the development of skin cancer are multiple and interrelated. Both endogenous factors such as genetic predisposition (i.e. skin type) and genetic diseases (i.e. xeroderma pigmentosum), as well as exogenous factors, are involved. The key environmental risk factor for NMSC is UV radiation from exposure to sunlight and artificial tanning lamps. Other exogenous factors include chemical carcinogens like arsenic, pesticides, hydrocarbons, such as tar, coal, paraffin, and certain industrial oils, dyes and solvents, ionizing radiation, several medical conditions, such as human papilloma virus infections, chronic irritation, scarring, hyperthermia, iatrogenic factors, such as immunosuppression especially in organ transplant recipients and intense PUVA therapy of more than 200 sessions. Tobacco smoking has also been recently linked to SCC.

However, the increasing skin cancer incidence is mainly attributed to the UV radiation that adults were exposed to at a young age. There is persuasive evidence that both BCC and SCC are caused by sun exposure. Skin cancer risk increases with increasing ambient solar radiation; the highest densities occurring on the most sun exposed parts of the body and the lowest on the least exposed. The risk is present in individuals with total (mainly SCC), occupational (mainly SCC) and non-occupational or recreational sun exposure (mainly BCC) and a history of sunburn and presence of benign sun damage on the skin.

People living at lower latitudes or those who spend extended periods of time outdoors or at high altitudes are at greater risk. However, while levels of total annual UV radiation vary approximately four-fold across the globe, in any area there is likely to be at least a ten-fold difference in personal UV radiation exposure, which is related to behavioral and cultural factors. Thus, even in areas of relatively low ambient UV radiation, it is possible to have high personal exposure. Cultural changes in the past 100 years have stimulated fair-skinned Caucasians to regard some degree of tanning as being cosmetically highly desirable.

Furthermore, availability of easy and inexpensive air travel has allowed white skinned populations resident in temperate climates to accumulate many more hours of sun exposure annually than was the case even 20 years ago. Additionally, we can postulate that in the EU, movement is not only observed for leisure purposes but that there is also an internal migration of citizens of the various member states. Thus a situation already exists whereby people may receive more intense and greater cumulative sun exposure than their genetic evolution has allowed for.

Apart from the ready access to natural UV radiation, the use of artificial sources of UV for tanning purposes has
become widespread and reached epidemic proportions in many parts of the world including Europe. With the increasing trend among adolescents, particularly girls, to use sun beds in an effort to look ‘healthier,’ the total lifetime UV exposure is increasing. There is evidence of a possible causal relationship between artificial UV radiation and NMSC but the lack of accurate data is a concern as the use of indoor tanning facilities appears to be increasing among the young.

An early study found a strong association (RR (relative risk) = 13.42) between the use of a long-tube sunlamp and SCC, but the risk estimate was based on small numbers of subjects. An American study showed an increased risk of SCC with the use of tanning lamps (OR (odds ratio) = 2.5; 95% CI (confidence interval) = 1.7–3.8) and a more modest risk for BCC (OR = 1.5; 95% CI = 1.1–2.1) Two other studies showed no association with sunlamps or tanning beds, but both studies had limited power due to low exposure prevalence. Once more we stress the need for well-conducted analytical studies with quantitative exposure data and acceptable controls for host susceptibility factors and concomitant sun exposure in order to clarify the risk and define appropriate interventions.

Primary and secondary prevention to fight global trends

Article 152 of the EC Treaty commits the Community to the promotion and improvement of health, the prevention of human illness and obviating sources of danger to human health. With the rise in NMSC incidence in Europe, emphasis should be placed in developing effective public health strategies to combat the disease at the Community level. This calls for coordinated and cohesive measures within the EU to ensure a high level of protection of public health. A sound public health policy adapted to the challenges of the 21st Century must strive to prevent skin cancer development through risk factor modification (primary prevention) and improved disease surveillance and earlier detection (secondary prevention).

The European Code Against Cancer recommends that ‘Care must be taken to avoid excessive sun exposure. It is specifically important to protect children and adolescents. For individuals who have a tendency to burn in the sun, active protective measures must be taken throughout life’. Widespread implementation of this recommendation would lead to a containment of the increase or even to a reduction in NMSC incidence, as has been demonstrated in Australia. The epidemiological data suggest that in implementing sun protection, an increase in intermittency of exposure should be avoided, that sun protection will have the greatest impact if achieved as early as possible in life and that it will probably have an impact later in life, especially in those who had high childhood exposure to solar radiation.

Adolescence is an important developmental period to introduce specific intervention programs aimed at reducing indoor tanning and sun exposure. Young people tend to discount health-related information, particularly when the information pertains to long-term consequences, because of a well-documented sense of personal invulnerability. In order to better target and engage the attention of adolescents, appearance-based campaigns focusing on the role of sun damage and tanning in skin ageing would perhaps be more effective. Secondary prevention through improved and earlier detection should also be promoted.

Once an individual develops an NMSC, there is an increased risk that a new skin cancer will appear within the next few years. The highest risk of a subsequent NMSC occurs within the first year. In a follow-up study, 52% of patients developed subsequent NMSC within five years of therapy of their first SCC. Therefore all patients with NMSC should be followed up for at least five years after treatment. Screening, in the form of a total body skin examination, is noninvasive, requires no special equipment and is reasonably cost-effective, compared to other conventional cancer screening strategies. Despite this, skin cancer secondary prevention practices are performed less frequently than other preventive practices, such as breast examinations, Papanicolaou tests, pelvic examinations and rectal examinations.

Skin examinations are rarely performed as part of routine primary care even for those patients in high-risk groups. Although routine screening examination of patients who are not at high risk for skin cancer is debated and there is a lack of direct evidence and consensus on its utility, early detection of skin cancer is critical in reducing morbidity and mortality. In addition to the lack of consistency on guidelines for skin cancer screening, physician attitudes toward skin cancer screening and prevention, as well as a lack of training, knowledge and clinical skills in the examination, have all been identified as barriers in clinical practice. A recent article by Moore et al. discussed physician preparation for performing skin cancer examination (SCE). By conducting a survey of students at seven renowned US medical schools the authors found low levels of observation, training and practice of the complete SCE, with most students rating themselves as unskilled in the examination. Only 28% of students rated themselves as somewhat or very skilled in the examination. Even small increases in training and practice opportunities, as well as elective exposures and hours spent in a dermatology clinic, were associated with higher self-rated SCE skill levels.

No such survey has been conducted in Europe. Acquiring basic clinical skills in the diagnosis and evaluation of suspicious skin lesions as well as skin cancer precursor lesions should be part of standard curricula at all medical schools. BCC arises de novo, which means there are no known precursor lesions. Precursor lesions for SCC include actinic keratoses (AKs) and Bowen’s disease. AKs usually occur in fair-skinned individuals over 45 years of age (skin type I or II). In lower latitudes, younger people are affected more frequently. Accurate figures on the prevalence and incidence are hard to obtain.

A recent study reported that the point prevalence of actinic keratoses (AK) in Italy was 1.4% (95% CI, 1.2%–1.8%).

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The prevalence was higher among men (1.5%) than women (1.4%). It increased with age from about 0.6% (45–54 years) up to 3.0% (>74 years). What is particularly interesting is the observation that 42% of patients with AK were unaware of their condition. The authors concluded that the prevalence of AK in Italy was considerably lower than that in the United States and Australia. Age, skin phenotype, and sun exposure appeared strongly associated with the prevalence of these lesions which also seemed to be under-diagnosed and undertreated in the population studied.

Approximately 5–20% of AK will transform into SCC within 10–25 years. It was calculated that the theoretical risk of malignant transformation for an average patient with AK followed up for 10 years would be 6.1% to 10.2%. By treating AK the development of SCC can be prevented. Secondary prevention of NMSC is extremely important because the prognosis improves substantially with early detection. Therefore, patients and physicians have to be educated how to detect skin cancers early, and unprotected exposure to UV light has to be decreased or eliminated.

Developing a European public health policy for NMSC

Europe’s ageing demographic and people’s growing expectations concerning healthcare are realities. Longevity is a sign of progress, but it comes at a price. Healthcare and public health represent approximately 10% of the Gross Domestic Product of the 25 EU Member States. Performing health-care systems, keeping the society healthy, active and productive are major challenges. It is clear that an older population in bad health can make healthcare budgets swell, but evidence shows that improved health in the overall population can greatly reduce projected increases in spending. Finally, tackling an ageing society also implies a lifestyle approach to health that ensures a focus on prevention strategies addressed at young people.

NMSC is the most common malignancy occurring in white populations and its incidence is increasing each year. These trends may be exacerbated by further increases in both acute and prolonged exposure to sunshine, together with increasing numbers of older people in the population. For the moment as mentioned above, we have no accurate data on these lesions at the Community level and as a result incidence rates are probably underestimated. In addition, the workload generated is also significantly underestimated by health economists and healthcare managers.

Developing a sound health knowledge base is essential if an evidence-based health policy towards NMSC is to be developed. This requires strategies of prevention, control and research. It is obvious we will only benefit by recording, collecting and analysing comparative data. By better understanding NMSC epidemiology in Europe we will improve prophylaxis by adapting it to modern trends of behaviour and making it more effective. Finally, by improving the skin examination training for physicians, developing guidelines and exchanging best practices on the treatment of these lesions, a high level of health services could be provided, thus preventing further health impairment.

Acknowledgments

Damiano Abeni is supported in part by funds from the ‘Progetto Ricerca Corrente’ of the Italian Ministry of Health.

References


Actinic keratosis is an early in situ squamous cell carcinoma: a proposal for reclassification

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Summary

The term actinic keratosis (AK) describes a sun-induced, clinical erythematous lesion covered with scale, but does not provide an understanding of the biology or histopathology of the lesion. Consequently, several classification systems for AK have been suggested, but as yet no consensus has been reached. These systems strive to correlate the pathological and clinical features to better provide physicians with the most accurate information to enable correct decisions to be made regarding treatments, Prognosis and metastatic potential. AK is a clinical description that has a histological diagnosis consistent with squamous cell carcinoma (SCC) in situ. We recommend an AK classification system that describes these lesions as squamous cell carcinomas (SCCs), using the terminology ‘early in situ SCC Type AK I’, ‘early in situ SCC type AK II’ and ‘in situ SCC Type AK III’, thereby giving clinicians better guidance for diagnosis and specific treatment recommendations.

Actinic keratosis (AK) is a common sun-induced skin lesion. The term AK describes a sun-induced, clinical erythematous lesion covered with scale, but it does not provide an understanding of the biology or histopathology of the lesion. Consequently, several new classification terms for AK have been suggested. Actinic keratosis has been described as a precursor of cancer or a precancerous lesion. In addition, it has been categorized as ‘keratinocyte intraepithelial neoplasia’ (KIN)1,2,3 and ‘proliferative actinic keratosis’ (PAK),4 and the concept of ‘inflamed actinic keratosis’ (IAK) has been put forward.5 These terms, however, still do not reflect the biological nature of these lesions. Actinic keratosis is a clinical description and is accepted to be the most frequent carcinoma in situ in man.6

We recommend an AK classification system that describes these lesions as squamous cell carcinomas (SCCs), such as the analogous classification proposed by Cockerell et al.1,2,3 In addition, the terminology early in situ SCC Type AK I and II, and in situ SCC Type AK III should be used to give clinicians better guidance for diagnosis and specific treatment recommendations.

Historical perspective

Actinic keratosis was first described in 1896 by Duhreuill7 who called this lesion ‘keratosis senilis’. He created the concept of pre-cancercerosis after finding evidence that keratosis senilis had a tendency to develop into invasive SCC of the skin. In 1926, Freudenthal described the histopathological features of keratosis senilis and believed that the lesion was a transition to cancer.8 Sutton postulated in 1938 that these lesions cannot be pre-cancerous as they are in fact already cancerous.9 In 1949, Lever proposed that keratosis senilis represents SCC Grade 1/2.10 It was Pinkus in 1958, who renamed the lesion AK in an attempt to better describe a keratotic (thickened, scaly) lesion caused by ultraviolet (UV) rays in sunlight.11 Historically, AK has been described as a pre-cancerous lesion.12 Many authors described these lesions as pre-malignant epithelial tumours that have the potential to develop into SCC.13–15 Other authors have postulated that there is no patho-biological difference between AK and SCC and that they represent a continuum of disease.16,17

Clinical appearance and biological behaviour

Actinic keratosis is usually characterised as scaly or keratotic, sometimes pigmented, papules with a diffuse erythematous base on sun-exposed skin, usually less than 1.0 cm in diameter. Actinic keratosis may be better diagnosed by the presence of palpation and a sand paper-like texture. The diagnosis of AK is usually made following clinical examination. However, based on the current histological classification of these lesions, no clear guideline exists to make the clinical distinction between AK and SCC. In one study, one in 25 AK lesions clinically diagnosed by a board certified dermatologist were found to actually
be occult early-stage SCC (based on the current classification system). These results confirm that it is difficult to clinically differentiate where AK ends and where SCC begins using the current classification system. We interpret this to mean that a distinction is difficult to make because there is in fact no difference between the two types of lesions.

Without treatment, AK can develop into invasive SCC and has the potential to metastasise and cause death. Based on clinical behaviour, AK is a malignant neoplasm. This progression is analogous to other malignancies, such as melanoma in situ developing into metastatic melanoma, adenomatous colon polyps developing into colon cancers, and abnormal cervical lesions developing into cervical carcinoma. Just as it is impossible to determine if and when a particular in situ carcinoma in each of these organ systems will progress to become an invasive carcinoma, it is impossible to determine whether AK will progress to invasive SCC.

Histological appearance

Histologically, AK is characterised by the presence of atypical keratinocytes at the basal cell layer of the epidermis, which in advanced lesions may extend into the entire epidermis. The epidermal keratinocytes of the acrosyringia and acrotrichia are spared and show a normal appearance and keratinisation, reflecting a normal orthokeratotic cornified layer. There are often small round buds at the basal layer which protrude into the papillary dermis. The maturation of the keratinocytes in the epidermis is defective resulting in parakeratosis alternating with hyperkeratosis. Acantholysis with subrabasal clefts may be seen. Actinic keratosis almost always has solar elastosis in the dermis and often shows an infiltrate of lymphocytes and plasma cells.

A SCC of the skin is a malignant neoplasm of epidermal keratinocytes. The thickened surface of the epidermis shows confluent parakeratosis and compact orthokeratosis. There is a loss of orderly maturation with atypical keratinocytes throughout the full thickness of the epidermis. The atypical keratinocytes have eosinophilic and sometimes pale or vacuolated, cytoplasm (a sign of faulty cornification) as well as whorls of parakeratosis within aggregations of neoplastic cells (horn pearls). There is an increased number of atypical mitotic and dyskeratotic, or necrotic keratinocytes throughout the epidermis. The nuclei of the atypical keratinocytes are crowded, pleomorphic and often large and hyperchromatic.

The histological and cytopathological changes seen in the individual cells of AK and invasive SCC are identical and not indistinguishable from one another. Both show atypical keratinocytes with loss of polarity, nuclear pleomorphism, disordered maturation and increased numbers of mitotic figures; many of them atypical and pleomorphic. It is not possible to tell where AK ends and SCC begins; for these reasons AK is an early in situ SCC.

Pathogenesis

Actinic keratosis is typically seen on chronic sun-exposed areas of the skin, particularly the face, arms, dorsum of the hand and upper back. High-risk populations include the elderly and people taking immunosuppressive therapy. Patients with AK usually have multiple lesions, reflecting the actinic damage to the field and forms the basis for the concept of field cancerisation. The most important cause of AK formation is UV-B radiation (wavelength 290–320) from sunlight. Specifically, UV-B radiation causes thymidine dimer formation in DNA and RNA, resulting in mutations. Of particular interest is the mutation in the p53 gene. The neoplastic transformed keratinocytes, which are present in over 90% of invasive SCCs, are also frequently present in AK. The tumour suppressor gene is located on chromosome 17p132 and leads to arrest of the cell cycle, which allows for repair of the damaged DNA. Dysregulation of the p53 pathways results in the unabated growth and proliferation of damaged keratinocytes and potentially neoplastic cells, and appears to be an early event in SCC carcinogenesis. These genetic mutations seem to be causally related to cellular transformation and are found in the earliest phase of skin cancer. Additionally, similar genetic aberrations have been described for SCC and AK. Other molecular markers that may indicate an increased likelihood of malignancy include the expression of p16\(^{\text{ink4}}\), the CD95 ligand, tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) and TRAIL receptors, and loss of heterozygosity (LOH).

Based on this, AK is actually an SCC. Actinic keratosis cannot evolve, transform or develop into a SCC because it is already an SCC. Actinic keratosis is neither a pre-cancer nor a precursor of cancer, but is cancer. Actinic keratosis and SCC are, therefore, two different names for the same process, with every AK being an in situ SCC.

Classification systems

Classifications of tumours are important. The aim of a classification system is to correlate the pathological and clinical features and provide physicians with the most accurate information to enable correct decisions to be made regarding treatment, prognosis and malignant potential.

The creation of a grading system for epithelial tumours, similar to that for other neoplasms, is therefore warranted and long overdue. Different classification schemes have been published that attempt to categorise AK, e.g. the term keratinocytic intraepidermal neoplasia (KIN) coined by Cockerell et al. Cockerell proposes three categories of KIN (KIN 1, KIN 2, and KIN 3), analogous to the cervical intra-epithelial neoplasia (CIN) and vulva intra-epithelial neoplasia (VIN) classification schemes of dysplasia in the cervix and vulva used to classify neoplastic lesions of the uterine cervix. These three categories, which are based on both clinical
features, as well as histological features (degree of cytological atypia of the epidermal keratinocytes and the extent of atypical keratinocytes in the epidermis), intend to better convey that lesions previously called AK are actually part of a disease continuum, which ends with invasive carcinoma.

This grading system for AK has value as it portrays exactly the histological appearance of the lesions and provides clinicians with an idea of the malignant risk of it developing into an invasive carcinoma. However, this system does not define what AK really is, i.e. an early in situ SCC.

The same argument holds true with the concept of PAK,4 that divides AK into two categories; proliferative and non-proliferative lesions, and IAK,5 which divides AK into three clinical groups with asymptomatic AK (AAK), inflamed AK (IAK) and SCC. Berhane et al5 hypothesised that the inflammatory infiltrate in inflamed lesions represents a cellular defence mechanism that if successful leads to lesion regression, and if unsuccessful to lesions transforming into invasive SCC. This is in contrast to Ackerman et al,26 who state that solar (actinic) keratosis is SCC, so that AK cannot convert or transform into SCC as it is already SCC.

A reclassification of AK

In an analogous fashion to Cockerell et al’s classification,2,3 we have categorised the AK histology into three types based on the extent of atypical keratinocytes in the epidermis. A drawback of Cockerell’s classification is that the term KIN is a description of a histopathological finding and could be used also for other epithelial neoplasia as seborrhoeic keratos, large cell acanthoma, pale cell acanthoma, Bowenoid papulosis and Bowen’s disease.

Additionally, it depends on a correlation of gross and histological findings, and also on the collaboration of clinicians and a pathologist to categorise these lesions. Such collaboration is not always feasible.29 However, because AK is a SCC we propose that it is called early in situ SCC. As we have found that there are varying extents of atypical keratinocytes in the epidermis of these lesions, we have divided the in situ SCC lesions into three categories according to Cockerell’s original proposal, i.e. Grade I (mild), Grade II (moderate) and Grade III (severe). We propose classifying AK as ‘early in situ SCC Type AK I’, ‘early in situ SCC Type AK II’ and ‘in situ SCC Type AK III’.

Early in situ SCC Type AK I (Grade I, mild)

In early in situ SCC Type AK I (Fig. 1), atypical keratinocytes are found in the basal and suprabasal layers of the epidermis. The nuclei are hyperchromatic, variable in size and have mild irregularities in nuclear outline. Often, a loss of nuclear polarity occurs, with many of the cells having oval nuclei oriented at obtuse angles instead of being perpendicular to the epidermis. The follicular infundibulum is uninvolved.

Early in situ SCC Type AK II (Grade II, moderate)

In early in situ SCC Type AK II (Fig. 2), atypical keratinocytes extend to the lower two-thirds of the epidermis, alternating with zones of normal epidermis (of the acrotrichia and acrosyringia in particular). Buds of keratinocytes in the upper papillary dermis can be found.

In situ SCC Type AK III (Grade III, severe)

In in situ SCC Type AK III (Fig. 3), atypical keratinocytes extend to more than two thirds of the full thickness of the epidermis and involves the epithelia of the hair follicle, infundibula and acrosyringia, as seen in SCC in situ. Buds of kera-
tinocytes in the upper papillary dermis can also be found. Grade III lesions are equivalent to those previously called SCC in situ.

In the circumstance where a single lesion has areas of various grades, the highest grade would define the lesion. In this classification system, AK is a clinical description that has a histological diagnosis and is based solely on the histological features. Because the gross clinical and histological features do not always correlate, we feel a system based on both these features is confusing and difficult to use routinely. Greater precision in the classification of these lesions will also allow for more accurate data collection in therapeutic and research studies.

Conclusions
As both AK and in situ SCC share the same clinical, histological and molecular features, it should be concluded that AK is an early in situ SCC. This is supported by recent publications that suggest AK is not a pre-malignant neoplasm but a SCC. It is generally known that AK can evolve into invasive SCC, however, it is not possible to predict if and which lesions will become invasive. Consequently, we support the view that all AKs must be treated to prevent possible invasion, metastasis and even mortality.

Currently, several AK classification systems exist. To our knowledge, we are the first to propose a system that classifies an AK as a SCC, in conjunction with an atypia grading system. We feel it is important to establish a new, simple classification/terminology that more accurately reflects the biology of the lesion. We therefore recommend using the terms ‘early in situ SCC Type AK I’, ‘early in situ SCC Type AK II’, and ‘in situ SCC Type AK III. We hope this delineation will give clinicians guidance regarding treatments. Without a grading system, the clinician does not have the tools to accurately judge the amount of atypical keratinocytes in the epidermis. We hope that in providing more information about the grade of AK, it will be possible to choose more precisely the appropriate therapy for these early SCCs.

Variations in efficacy previously reported with AK therapies are possibly related to ambiguity in the histological definition of AK. Further studies are necessary to delineate which therapies are effective based on this new classification system.

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Actinic keratoses: non-invasive diagnosis for field cancerisation

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Summary

Background Actinic keratoses (AKs) are among the most common cutaneous malignancies and have previously been classified as in situ squamous cell carcinoma (SCC) with reported progression rates of up to 20% over 10 years. Since current scientific evidence suggests the presence of multilocular preneoplastic changes in the areas surrounding the affected skin sites, the detection of subclinical AKs remain an ongoing and challenging effort in the clinical and diagnostic management of these lesions. In vivo reflectance confocal microscopy (RCM) has been used for evaluation of the morphological features of non-melanoma skin cancer (NMSC) and RCM evaluation parameters for the diagnosis of AKs have been reported.

Objectives The objective of this study was to evaluate the RCM-morphology of clinically diagnosed AKs in our study population and to correlate the findings with routine histopathology.

Patients/methods Forty four Caucasians (SPT I-III) with a minimum of one actinic keratosis (AK) lesion were included in this study. Evaluation consisted of clinical examination, RCM and routine histology. Reflectance confocal microscopy evaluation parameters included parakeratosis, architectural disarray and keratinocyte pleomorphism.

Results A total of 44 AKs were included in the final analysis. Following blinded evaluation by two independent investigators, 97.7% of all skin samples were identified as AK using RCM. 2.3% were incorrectly identified as normal skin by RCM, while routine histology showed features consistent with AK.

Conclusions Reflectance confocal microscopy may be a feasible alternative in the diagnosis of AK and may aid in the differentiation against normal skin, as well as in the detection of subclinical disease.

Actinic keratoses (AKs) are among the most common cutaneous malignancies and have previously been defined as in situ squamous cell carcinoma (SCC) with reported progression rates of up to 20% over 10 years. Implicated risk factors include a history of long-standing sun-exposure, chronic immunosuppression, skin phototypes I-III, PUVA-therapy, arsenic exposure and chronic cutaneous inflammation. The concept of ‘field cancerisation’ suggests that the clinically normal appearing skin around AKs and SCCs provides the basis for clonal expansion of genetically altered neoplastic cells.

Actinic keratoses are presented clinically as erythematous, hyperkeratotic papules or patches in sun-exposed areas of the face, forearms, hands and lower legs and are often better felt than seen. Although they may occur as single lesions, multiple AKs are commonly present in areas of chronic actinic damage. Histologically, AKs have been classified according to keratinocyte atypia, nuclear pleomorphism, hyperkeratosis, parakeratosis, a dermal inflammatory infiltrate and concomitant solar elastosis. Due to its similarity in biological behaviour and comparable progression rates to other intraepithelial malignancies, several investigators have proposed the concept of AKs as keratinocyte intraepithelial neoplasia (KIN) with subdivision into three histomorphological grades. In grade I disease, the neoplastic changes are limited to the lower third of the epidermis, whereas in grade II, the lower two thirds of the epidermis is involved, and in grade III full-thickness atypia is found.

Currently, the diagnosis of actinic keratosis (AK) is made on clinical grounds, yet routine histopathology remains the
gold standard. Data on the diagnostic accuracy of clinical diagnosis are scarce. A study comparing the clinical diagnosis of classic AK by a board certified dermatologist with histopathological examination showed a positive predictive value (PPV) for clinical diagnoses of 74%. In the same study, histopathology was read by two independent dermatopathologists and showed 100% concordance between the two investigators.6 Other trials have reported between 80–94% confirmation of clinical diagnosis by histopathology.7–8 A recent study by Ehrig et al.9 showed a histo-clinico-pathological correlation of only 91%, suggesting that one in 25 lesions have already progressed to either a SCC or actually represented a different histopathologic entity. Since the majority of patients present with numerous AKs at various stages of graded differentiation, the extent of subclinical disease is particularly difficult to determine.

Thus, when considering current diagnostic modalities, it becomes evident that a diagnosis based on clinical grounds alone may not always be sufficiently reliable, while the feasibility of obtaining biopsies at all affected and surrounding skin sites is limited. Hence, in recent years interest has broadened to develop non-invasive diagnostic modalities to detect not only clinical AKs but also to detect and define subclinical disease.

In vivo reflectance and fluorescence confocal microscopy (CM) generate horizontal skin sections at a resolution comparable to routine histology. Reflectance confocal microscopy (RCM) has previously been used for the evaluation of histopathological features of non-melanoma skin cancer [NMSC; AK and basal cell carcinoma (BCC)] and may be the most promising method for real-time evaluation of cutaneous tissue. The diagnostic criteria of AK using RCM have previously been described by Aghassi et al.10

The aim of this study was to evaluate the RCM-morphologic features of clinically diagnosed AKs and to correlate the findings with routine histopathology.

Materials and methods

Study population

A total of 44 Caucasians with a minimum of one AK participated in this study. Written informed consent was obtained from each study participant. Evaluation consisted of clinical examination, RCM and routine histology and a total of 44 AKs were included in the final analysis. Reflectance confocal microscopy evaluation parameters included parakeratosis, architectural disarray, keratinocyte atypia and pleomorphism and are shown in Table 1. Clinical evaluation was performed on all patients (n = 44) and one skin lesion suspicious for early, low grade AK was selected for RCM analysis and routine histology.

Clinical evaluation

A total body examination was performed in each patient and based on clinical evaluation, one lesion suggestive of early, low grade AK was identified for imaging. Clinical photographs of the skin lesions were taken in all study participants using a digital camera (Nikon Coolpix 950, Nikon Corp., Tokyo, Japan) under standardised conditions (Fig. 1). A total of 44 sites suspected to have AKs were selected for RCM imaging.

In vivo RCM evaluation

A commercially available in vivo RCM (Vivascope 1500, MAVIG GmbH, Munich, Germany) was used to image AKs

<table>
<thead>
<tr>
<th>Epidermal level</th>
<th>Evaluation criteria</th>
<th>RCM</th>
<th>Routine histology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stratum corneum</td>
<td>Disruption/individual cells</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td></td>
<td>Parakeratosis</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Alternating orthokeratosis</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Hyperkeratosis</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td></td>
<td>Impetiginisation</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td>Stratum granulosum/spinosum</td>
<td>Architectural disarray</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Cellular/nuclear polymorphism</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Spongiosis</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td></td>
<td>Exocytosis</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td></td>
<td>Mitosis/atypical mitosis</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Dermis</td>
<td>Solar elastosis</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Increased vascularity/blood vessel dilatation</td>
<td>+</td>
<td>+/-</td>
</tr>
<tr>
<td></td>
<td>Lymphocyte rolling</td>
<td>+/-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Superficial inflammatory infiltrate</td>
<td>+/-</td>
<td>+/-</td>
</tr>
</tbody>
</table>

RCM: reflectance confocal microscopy.

Table 1: Evaluation criteria for AKs for RCM and routine histology

The diagnostic criteria of AK using RCM have previously been described by Aghassi et al.10
and the respective control skin sites. A detailed description of this technique and the device used has been published previously.11–13 In each participant, one lesion that was clinically suspected for early AK was selected for imaging. For each skin site analysed, systematic horizontal mapping was performed and 4–6 images were captured in axial sections beginning with the stratum corneum, through the entire epidermis, and into the upper reticular dermis. Reflectance confocal microscopy images were individually subjected to evaluation.

Diagnostic parameters of AKs were as described previously.10 Evaluation parameters are shown in Table 1. An investigator obtained images of all test sites. The sites were systematically evaluated for the presence or absence of individual RCM features and a score of 1 or 0 was assigned to each respective parameter.

The corresponding RCM images of 44 AK sites were then subjected to blinded evaluation by two independent experts in the field of confocal microscopy. The assessors were blinded to participant name, age and suspected diagnosis and were given coded images in random for assessment. Prior to the scoring, the assessors were given instructions for the interpretation of RCM images by an expert in the field.

Routine histology
Skin punch biopsies (4 mm in diameter) were obtained from all 44 skin sites suspected to have AKs upon completion of clinical and RCM evaluation. The skin samples were fixed in formalin and processed by routine histopathology. Staining with haematoxylin and eosin was performed and the slides were evaluated by a board-certified dermatopathologist. Using previously defined criteria,5 the AKs neoplastic changes were classified into 3 grades. Actinic keratosis with atypia in the lower third of the epidermis was defined as grade I, atypia of the lower two thirds as grade II and full thickness neoplastic changes as grade III.

Results
A total of 44 skin sites were included in the study. All biopsy samples suspected of having AKs were received and processed by our laboratory between 30th August 2005 and March 30th 2006. Approximately 97.7% of all lesions were correctly identified as AK by RCM evaluation, whereas 2.3% were incorrectly described as normal, unaffected skin samples. Evaluation criteria consistently visualised by RCM in the presence of actinic changes included keratinocyte atypia, nuclear and cellular pleomorphism, hyperkeratosis, parakeratosis, inflammatory cells, blood vessel dilatation and solar elastosis. Representative RCM images are shown in Figures 2 and 3.

Routine histopathology examination of the skin samples revealed that 30/44 (68.2%) lesions had grade I dysplasia, 7/44 (15.9%) lesions had grade II dysplasia and 7/44 (15.9%) lesions had grade III dysplasia. These results are as shown in Table 2.

Discussion
The concept of ‘field cancerisation’ suggests that subclinical preneoplastic changes are frequently if not always present in skin sites surrounding the AK. Recent scientific evidence supports the presence of contiguous fields of preneoplastic keratinocytes, which genetically are clonal in origin. When considering current diagnostic modalities, it becomes clear...
that a diagnosis based on clinical grounds alone may not always be sufficiently reliable, while the feasibility of obtaining biopsies of all affected and surrounding skin sites is limited. Hence, in recent years interest has broadened to develop non-invasive diagnostic modalities not only to visualise clinical AKs, but also to detect and define subclinical disease.

Yet while histopathology remains the gold standard, with respect to the often widely affected skin sites, repeated biopsies may not always represent a practicable approach to the diagnosis and management of these lesions. It has therefore repeatedly been investigated whether non-invasive diagnostic tools represent a feasible alternative to routine histology, and whether adjunct optical technologies may enhance accuracy of clinical diagnosis. Among those, in vivo RCM has previously been used for real-time evaluation of the histopathological confocal features of NMSC, especially for the non-invasive diagnosis of BCC.\textsuperscript{14–17} However, the evaluation of AKs has only been the subject of preliminary studies.\textsuperscript{10} The goal of this investigation was to determine the clinical applicability of CM to aid in the diagnosis of AKs, and to confirm the clinical diagnosis with a non-invasive imaging technique with near cellular resolution and consecutive correlation to routine histology.

The findings presented herein support previous observations that RCM may bridge the gap between clinical evaluation and histopathologic evaluation in that it allows tissue analysis at near cellular resolution that is comparable to routine histology. A total of 97.7% of all lesions in this study were correctly identified as AKs, while 2.3% were misdiagnosed as normal skin. The incorrect diagnosis may be explained by the current limitation of RCM technology which does not allow full resolution of lesions with thick hyperkeratosis. The high refraction index of a thick hyperkeratotic stratum corneum does not allow sufficient penetration to reliably evaluate the cellular architecture underneath. By the same token, other parameters such as inflammatory infiltrate or exocytosis may not be consistently visualised and thus may not represent relevant parameters for the characterisation of AKs using RCM.

Furthermore at the present time, the horizontal sections obtained by RCM do not permit the detection of vertical invasion of individual lesions on horizontal sections; yet this study was not aimed at differentiating AKs (in situ SCCs) from invasive SCCs. Our findings demonstrate the potential of RCM to detect morphological features of AKs in correlation with routine histology.

Interestingly, out of the 44 clinically mild to moderate appearing AKs that were analysed in the study, 7 (15.9%) were histopathologically described as grade III AKs with full thickness dysplasia of the epidermis. These findings are consistent with other investigations that have shown that clinical assessment may not always be reliable.

### Table 2 Histopathologic grading of AKs

<table>
<thead>
<tr>
<th>Grading of AKs</th>
<th>Histopathology of AKs</th>
<th>Number of AKs</th>
<th>Percentage of AKs (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade I (KIN I)</td>
<td>Neoplastic cells in the lower third of the epidermis</td>
<td>30</td>
<td>68.2</td>
</tr>
<tr>
<td>Grade II (KIN II)</td>
<td>Neoplastic cells in the lower two thirds of the epidermis</td>
<td>7</td>
<td>15.9</td>
</tr>
<tr>
<td>Grade III (KIN III)</td>
<td>Full thickness epidermal atypia</td>
<td>7</td>
<td>15.9</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>44</td>
<td>100</td>
</tr>
</tbody>
</table>

AKs: actinic keratoses.
KIN: keratinocyte intraepithelial neoplasia.
Table 2 illustrates the different histopathologic grades of AKs in the study population.
The therapeutic management of AK includes cryotherapy, chemical peels, CO₂ laser resurfacing and a number of topical treatments, e.g. 5-fluorouracil, imiquimod and diclofenac 3% gel. The latter is an inhibitor of the arachidonic acid pathway and was introduced in Europe in 2001 for the management of AKs. Based on the visible clinical response of previously subclinical lesions under therapy, the concept of ‘field cancerisation’ has been established, suggesting the neoplastic transformation of an entire ‘field’ of atypical keratinocytes, rather than the presence of selected focal lesions only. This has to be taken into account when new therapeutic strategies are being evaluated for their efficacy and safety.

In summary, these preliminary findings suggest that RCM may be a useful non-invasive tool for detecting AKs. This tool may be a feasible alternative to clinical diagnosis alone and could aid in the differentiation of AKs against normal skin, as well as in the detection of subclinical disease. In the context of ‘field cancerisation’, repeated and multiple biopsies may not always be considered a practical approach. Therefore, evidence suggests that RCM may be a promising tool for the non-invasive evaluation, diagnosis and monitoring of AKs. Future studies are warranted to test the clinical applicability of our findings.

Acknowledgments

This study was supported by departmental funds and the authors would also like to acknowledge MAVIG GmbH for providing the Vivascope 1500 for this study.

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Apoptosis pathways as promising targets for skin cancer therapy


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Accepted for publication
5 March 2007

Key words
apoptosis, Bcl-2, COX-2, skin cancer, TRAIL

Conflict of interest
C. Ulrich has acted as a lecturer for Shire Pharmaceuticals. E. Stockfleth has acted as a lecturer/consultant for Shire Pharmaceuticals. All remaining authors declare no conflict of interest.

Summary

Apoptosis pathways provide efficient safeguard mechanisms against cancer that are mediated via cell-intrinsic responses and immune-mediated extrinsic signals. Intrinsic pro-apoptotic pathways are largely controlled by p53 and Bcl-2 proteins, whereas the extrinsic induction of apoptosis is initiated by death ligands, such as tumour necrosis factor-alpha (TNF-α), CD95L/FasL and TNF-related apoptosis-inducing ligand (TRAIL), or by granzyme B. Initiation of these pathways results in the induction of a caspase cascade leading to cell death.

The inactivation of pro-apoptotic pathways is elementary for tumourigenesis and may be responsible for therapy resistance. Thus, apoptosis-based strategies represent important tools for the development of effective tumour therapies. The aim of these therapies is to restore p53 activity, downregulate anti-apoptotic Bcl-2 proteins or NF-κB activity, and to upregulate extrinsic, death receptor-mediated pathways. The initial results of apoptosis-based strategies are proving promising. Also, topical treatments for actinic keratosis (AK), such as cyclo-oxygenase-2 inhibitors (e.g. diclofenac 3% gel), have been shown to trigger pro-apoptotic pathways. There is hope that pro-apoptotic strategies will lead to pronounced therapeutic success against skin cancer. Importantly, the involvement of the different pro-apoptotic pathways in specific tumour types needs to be unravelled and understood in order to evaluate drug effectiveness, as well as to modify and optimise therapeutic approaches.

UV light protection

The skin provides an effective barrier against environmental stress, including toxic chemicals and ultraviolet (UV) light. The direct link between exposure to UV light and skin cancer is now well established and as people are spending more time in the sun, the incidence of skin tumours has dramatically increased over the last decades. Overexposure to UV light damages DNA of skin cells. Ultraviolet is absorbed by the nucleotides resulting in chemical changes to particular pyrimidine dimers. Upon subsequent cell divisions, these mutations can persist in the genetic code of the daughter cells.

Trillions of cells must coexist together in order to ensure normal development and maintenance of the human body. As yet, only poorly understood mechanisms mediated by molecular regulators (e.g. cytokines, receptors, kinases, transcription factors) control for this coexistence by maintaining a fine balance between cell proliferation, cell differentiation and cell death. Thus, programmed cell death (apoptosis) is an important process in the maintenance of cell/tissue homeostasis within the body. In healthy individuals, there is a harmonious coexistence of cells, whereas in disease states, cell homeostasis is disturbed. In cancer, the regulators of cell division (cell cycle) and apoptosis are particularly affected.

Over the course of evolution the human body has evolved efficient safeguard mechanisms to prevent cancer. Some areas of the body, mainly the scalp, are covered in hair, which provides an effective protection against UV light. Areas of skin more exposed to UV light are protected by skin pigmentation. The skin pigment, melanin, is synthesised by epidermal melanocytes in response to exposure to UV light and then passed to the surrounding keratinocytes. Melanin absorbs UV light and presents an efficient barrier. The effectiveness of melanin to protect against the effects of UV light is highlighted by the increased incidence of skin tumours in people that suffer from albinism, a hereditary disease characterised by loss-of-function mutations for enzymes of the melanin synthesis pathway, e.g. the key enzyme tyrosinase.

The next level of protection against cancer is represented by a number of cellular DNA repair enzymes, which continuously safeguard the integrity of the genetic information in cells. The essential safeguard function against skin cancer, provided by these enzymes, is highlighted in xeroderma pigmentosum. This is a human hereditary disease, which is characterised by...
homzygous mutations leading to deficiencies in single DNA repair enzymes. The prevalence of these types of mutations is relatively high (up to 1:200), but fortunately they are recessive and there are a number of complementation groups. Patients with xeroderma pigmentosum are at increased risk of a variety of malignancies, in particular skin cancer.4

Cell-intrinsic pro-apoptotic tumour defences

If DNA damage is not repaired sufficiently, or rapidly enough (before replication occurs), further intrinsic cellular control mechanisms are initiated. These can be activated by cellular damage, in particular to DNA. Among the first responses is the stoppage of cell division. This prevents the propagation of mutations to daughter cells and possibly provides time for DNA-repair. If however, the DNA damage cannot be repaired, intrinsic control mechanisms induce apoptosis.5 A critical regulator in these pathways is the tumour suppressor and transcription factor p53. The importance of this intrinsic defence against cancer becomes apparent when faced with the high incidence of cancer in patients with Li Fraumeni syndrome, an inherited disease with mutated p53.6

Greater DNA damage or repeated UV exposure increases the risk of genetically changed cells occurring, and the risk that intrinsic control mechanisms may be overcome. Some of the genetically changed cells may possess the potential for malignant transformation. This may be due to mutations leading to inactivated tumour suppressor genes or activated oncogenes that enable these cells to overcome the intrinsic apoptosis control steps. Thereby, affected cells can gain time for the accumulation of further mutations than can be transmitted to their daughter cells. Consequently, higher malignant cells can arise progressively. This may also partly explain the typically long tumour development times, e.g. for malignant melanoma. Once the intrinsic mechanisms have been circumvented only immunological (extrinsic) defences may help to prevent tumour progression.

Immunological (extrinsic) tumour defences

In the course of vertebrate evolution, a number of highly elaborated tumour defence systems have emerged, based on the diverse activities of the immune system. Thereby, humoral defence can be distinguished from the cell-bound defence mechanisms. Antibodies produced by B-lymphocytes can be directed against bacterial or viral antigens but may also target some tumour antigens.7 Antibodies bound to target cells activate the complement system with the final goal of tumour cell lysis.

The cell-bound immunological tumour defence is supported by cytotoxic T-lymphocytes and natural killer (NK)-cells. An important prerequisite for recognition by T-lymphocytes is a kind of ‘identity card’ presented by each cell. This consists of fragments of the cells own proteins bound to the major histocompatibility complex (MHC) on the cell surface. Outside of the cell, there is an army of mostly short-lived and continuously renewed lymphocytes, which are characterised by an enormous multiplicity of different receptors, resulting from immunoglobulin rearrangement. These lymphocytes permanently screen the cellular ‘identity cards’ and cells with unknown protein fragments can be detected. This helps for elimination of virus-infected cells (viral proteins) and may also help against cancer cells (mutated proteins). The process is based on selection of T-lymphocyte clones, which carry a rearranged T-cell receptor that fits to the identified antigen.8 The elimination of the target cells employs pro-apoptotic signals delivered to the target cells by activated T-lymphocytes as well as by NK cells (see below). A clear impression about the significance of tumour defence by the immune system is provided by the dramatically increased incidence of skin tumours in immunosuppressed transplant patients.9 (See Ulrich et al., p. 40)

Apoptosis pathways – initiation phase

The regulation of apoptosis is important for embryonic development and tissue homeostasis, and is a necessary component of the intrinsic, as well as extrinsic tumour defences. Apoptosis has been recognised as a special form of cell death and was first described in 1972.10 As an active process (‘suicide program’ of the cell), it can be distinguished from necrosis, the ‘death by accident’ of the cell. Apoptosis is characterised by clear morphological changes, such as chromatin condensation, shrinkage of the cell and nuclear fragmentation. In addition, there is a sequence of biochemical changes which results in typical DNA fragmentation (Fig. 1). Owing to the key function of apoptotic processes in the fate of cells, the related signal pathways are subject to a fine and often repeatedly controlled regulation network (Fig. 2). These options for counter-regulation necessary for the survival of normal cells, present multiple possibilities for tumour cells to avoid apoptosis.

Fig 1. Characteristics of apoptosis. a: Nuclear morphologies of apoptotic melanoma cells (fluorescence microscope after DNA staining with Hoechst dye), b: DNA fragment ladder after induced apoptosis (+) as compared to the control (−).
The intrinsic signal pathway of apoptosis especially employs the level of the mitochondrial membrane. Activated by cellular damage, the signal pathway starts with the activation of kinases, such as ATM (ataxia telangiectasia, mutated) or ATR (ataxia telangiectasia, Rad3-related) or acetyl transferases. The modification of the transcription factor p53 by these enzymes results in its stabilisation, whereas under normal conditions, p53 is quickly degraded by the proteasome pathway initiated by its negative regulator, the ubiquitin ligase Mdm-2. As a consequence of p53 stabilisation, the expression of various p53-regulated proteins is induced. These include cell cycle inhibitors, such as p21, which initially stop the cell cycle, providing time for repair mechanisms to have an effect. In the presence of irreparable damage, p53 activation results in the transactivation of pro-apoptotic factors, in particular transactivation of the family of pro-apoptotic Bcl-2 proteins, such as Bax, Bik/Nbk, Noxa and Puma. Inhibition of the cell cycle may be necessary for the efficient induction of apoptosis, as both mechanisms exhaust the cells energy resources and are thus mutually exclusive.

The superfamily of over 20 identified Bcl-2 proteins decisively controls the mitochondrial signalling pathway. They are categorised according to the presence of four known Bcl-2 homology domains (BH1–BH4), as well as their pro- or anti-apoptotic functions. Most anti-apoptotic proteins, e.g. Bcl-2 or Bcl-x, share all four BH domains. On the other hand, the pro-apoptotic Bcl-2 proteins are subdivided into multidomain proteins, such as Bax and Bak, as well as a subfamily of BH3-only proteins. Most multi-domain proteins lack only the BH4 domain, whereas the BH3-only proteins (e.g. Bid, Nbk/Bik, Noxa and Puma) share exclusively BH3. The Bcl-2 proteins are active in the outer mitochondrial membrane, where they control the formation of pores/channels, through which several mitochondrial factors (e.g. cytochrome C, endonuclease G, apoptosis-inducing factor, SMAC/DIABLO, HTRA1/Omi) are released into the cytoplasm. In the mitochondria these factors exert specific mitochondrial functions, but in the cytoplasm they possess pro-apoptotic activities. Thus, the mitochondrial release of pro-apoptotic proteins appears to be a critical step, which is dependent on the equilibrium of pro- and anti-apoptotic Bcl-2 proteins in the mitochondrial membrane. Pro-apoptotic Bcl-2 proteins support the pore formation while the anti-apoptotic proteins try to block it.

Of central importance is the pro-apoptotic function of cytochrome C, which is an essential constituent in the mitochondrial respiratory chain in healthy cells. In the cytoplasm, it induces the formation of a multi-protein complex (apoptosome), which consists of cytochrome c, the adapter protein Apaf-1, adenosine triphosphate (ATP) and procaspase-9. The special three-dimensional structure of this complex results in activation of initiator caspase-9, which initiates a subsequent caspase cascade. Caspases (aspartate-specific cysteine proteases) are cysteine proteases, which cleave target proteins after the amino acid aspartate. Typically, caspases activate each other in a specific order, thus forming a signal cascade.

The other big complex of pathways is activated by extrinsic signals, such as granzyme B and death ligands. Caspases are also of critical significance for these extrinsic pathways. The secretion of the protease, granzyme B, and the presence of death ligands of the TNF-α family is a prerequisite for the extrinsic induction of apoptosis by cytotoxic T-lymphocytes and NK-cells. There are six death receptors as well as several decoy receptors, of which the receptors for TNF-α, CD95L/FasL and TRAIL are well described (Fig. 3). Death receptors are able to activate the pro-apoptotic caspase cascade as well as the NF-κB pathway. As NF-κB itself mediates the transactivation of several anti-apoptotic factors, it may enable a cell to initiate a more balanced response.

The binding of death ligands to their receptors leads to receptor multimerisation and formation of a membrane-bound protein complex, known as the death-inducing signaling complex (DISC), which activates the proapoptotic initiator caspas-8 and -10. A typical feature of cellular signal pathways is the diversity and multiple branching, which may allow a
range of cellular responses to occur in response to the diversity of ingoing signals. Important cross-connections in apoptosis pathways are represented by the transactivation of death ligands and death receptors by p53, as well as by the cleavage and activation of the pro-apoptotic Bcl-2 protein, Bid, by caspase-8. Upon cleavage, Bid transfers into the mitochondrial membrane, and here it can activate the mitochondrial pathway via Bax or Bak (Fig. 2). Via these cross-connections, one or the other signal pathway can be enhanced.

Apoptosis pathways – execution phase

The initiation phases of the extrinsic and intrinsic apoptosis pathways lead to the activation of initiator caspases (caspase-8, -9, or -10). These can subsequently activate effector caspases, such as caspase-3, -6 and -7 through proteolytic cleavage steps. Effector caspases, in turn, target a variety of cellular proteins (death substrates), including DNA repair and modifying enzymes, and various signalling and structural proteins. Many death substrates are inactivated by cleavage while others are activated upon caspase activity.

A typical example of a protein that is inactivated through caspases is the DNA repair enzyme PARP (poly ADP-ribose polymerase). DNA repair is no longer required in apoptotic cells, and even it competes with the apoptotic process, as both use cellular energy resources (ATP). The cleavage of PARP thus guarantees that enzymatic repair is stopped and that energy resources are reserved for apoptosis.

A typical example of an enzyme activated by caspase activity is caspase-activated DNase (CAD). In healthy cells, this nuclease is inhibited by the binding of ‘inhibitor of CAD’ (ICAD). After apoptosis is induced, caspase-3 cleaves and inactivates ICAD leading to free CAD, which starts to cleave the DNA strands between each two nucleosomes, resulting in the characteristic DNA fragment ladder (Fig. 1). The protease granzyme B, secreted by cytotoxic T-lymphocytes and NK-cells, contributes at several steps of the caspase cascade, thus supporting cell death mediated by death ligand in the target cells. It has been shown that granzyme B, which penetrates through the cytoplasmic membrane of target cells with the assistance of perforin, in a similar way to caspases, is capable of cleaving Bid, caspase-3 and/or ICAD. Through the combined activities of proteases and death substrates, the cell is finally completely reprogrammed, and apoptosis is implemented.

Apoptosis resistance in tumours

In this context, the fundamental significance of apoptosis in the intrinsic and extrinsic/immunological defences against skin tumours becomes apparent. For therapeutic considerations an important question arises; why do skin tumours develop despite the presence of these elaborate protection mechanisms? Tumour research over the last few decades has revealed that the acquired apoptosis resistance of tumour cells provides an answer to this question. Tumour cells contain multiple genetic changes and cellular dysfunctions, which would activate intrinsic apoptosis pathways in normal cells leading to their destruction. In addition, most tumour cell clones are characterised by an unusual expression of proteins and may contain altered proteins that should attract the attention of the immune cells. Indeed, immune responses occur in many skin tumours as demonstrated by frequently high numbers of tumour-infiltrating lymphocytes (Fig. 4).

The main causes for the apoptosis resistance of tumour cells are defects in the pro-apoptotic signal pathways. Thus, blockage of the intrinsic signal pathways is widespread in most tumours. The transcription factor, p53 is the most frequently mutated tumour suppressor gene and is turned off in about half of all tumours, particularly in epithelial cancer. For basalioma and cutaneous squamous cell carcinoma (SCC), mutation
rates of roughly 50–60% have been reported, illustrating the great importance of this signal pathway in skin tumour prevention. Mutations of p53 have also been found at a high frequency in actinic keratosis (AK) and the presence of pyrimidine dimers were clearly indicative of UV effects.27,28 As many chemotherapeutics also exert their effects via this pathway,29 p53 mutations may further contribute to multiple therapy resistance.

Selective blocking of extrinsic signals in several tumours may result from a loss of death receptors. Thus, lymphocytic tumours, such as B- and T-cell lymphomas are frequently characterised by loss of CD95.30 Loss of the agonistic TRAIL receptor, TRAIL-R1/DR4, has been found in melanoma cell lines associated with reduced sensitivity or resistance.31 Besides this, the expression profile of protein factors, which exert their function in the DISC, may change and therefore block the extrinsic pathways. A well established example is the Flice-inhibitory protein (FLIP), which exerts competitive inhibitory activity against caspase-8. Flice-inhibitory protein has been found to be overexpressed in several lymphocytic and solid tumours, and its overexpression appears to be a typical feature especially in Hodgkin lymphoma.32

In many tumour cells, the main pathways of apoptosis converge at the mitochondrial level. Thus, Bcl-2 proteins exert a decisive role in apoptosis control. A shift of the balance between pro- and anti-apoptotic Bcl-2 proteins can, therefore, lead to a blockage of both main pro-apoptotic pathways. A correlation has been found in melanoma cells between apoptosis sensitivity and the expression ratio of pro- to anti-apoptotic Bcl-2 proteins.33

In the final phase, apoptosis pathways lead to the activation of effector caspases. At this stage, several specific protein inhibitors of caspases, e.g. inhibitors of apoptosis proteins (IAPs) come into play, which can bind to activated effector caspases or to initiator-caspase-9, and can block their activity. Upregulation of IAPs has been described for different tumours, including melanoma and SCC.34 Also, several anti-apoptotic programmes trace back to the activities of the transcription factor nuclear factor-kappaB (NF-κB), which besides its important pro-inflammatory functions drives the expression of some anti-apoptotic protein factors of the Bcl-2 and IAP families. Constitutively high NF-κB activities have been described in different tumours and have been associated with apoptosis resistance.35

### Apoptosis-based therapeutic approaches

In principle, the final goal of the majority of chemotherapies, as well as of hormonal or radiotherapies, is the induction of apoptosis in tumour cells. In addition, there are a significant number of new therapeutic concepts, which directly target pro- or anti-apoptotic signalling pathways in cancer cells.36,37 High Bcl-2 expression in melanoma cells gave rise to therapeutic strategies based on antisense molecules against Bcl-2 (Oblimersen). The potential of this strategy was proven in vitro as well as in animal studies, and clinical trials have been started.38,39 Although first results may indicate that an anti-Bcl-2 strategy alone may not be sufficient for metastasised melanoma,36 it is important to keep in mind that the Bcl-2 family includes several anti-apoptotic factors, which may outweigh the activities of Bcl-2. Combined strategies simultaneously against several Bcl-2 proteins are presently being tested.40 Complementary to the downregulation of anti-apoptotic Bcl-2 proteins may be the overexpression of pro-apoptotic Bcl-2 proteins.41,42 Other new strategies are based on peptides or small molecules which mimic the pro-apoptotic BH3 domain of the Bcl-2 proteins.36

The significance of the transcription factor, NF-κB in the apoptosis resistance of tumour cells forms the basis for therapeutic strategies with proteasome inhibitors, which delay the degradation of the NF-κB inhibitor (IκB).43 Thereby, the NF-κB-driven expression of anti-apoptotic factors is blocked, including Bcl-XL, as well as caspase inhibitors, cIAP-1, cIAP-2, XIAP and FLIP.44 The effectiveness of proteasome inhibitors in several tumour types is currently being evaluated in clinical trials.45

Apoptosis may be directly induced in cancer cells by death ligands, as has been shown by the overexpression of CD95L in melanoma cells and melanoma mouse models.46,47 The death ligand, TRAIL, is particularly appealing because of its property to selectively induce apoptosis in tumour cells, while many non-malignant cell types are largely protected.48,49 The benefit of TRAIL therapy has been demonstrated in suitable animal models either as monotherapy or in combination with chemotherapeutics.50,51 In addition, agonistic TRAIL receptor antibodies have been developed, which have shown comparable efficacy. Clinical trials are underway in patients with lung cancer, non-Hodgkin lymphomas and colorectal cancer.52
Since the p53 gene is the most frequently mutated tumour suppressor gene and often inactivated in epithelial cancer, it has attracted a lot of attention as a possible target in tumour therapy. Gene-therapeutic strategies with adenoviral vectors carrying the wild-type allele have been developed (gencicine) and are being evaluated in several clinical trials for the treatment of oesophageal, SCC and breast cancer.

For many cutaneous epithelial cancers, especially AK and/or field cancerisation in immunosuppressed patients, topical treatments may evolve the most suitable therapeutic strategies. The first topical approach in immunosuppressed patients used imiquimod, a potent toll-like receptor-dependent immunomodulator. Besides the dominant immunomodulatory effects of imiquimod, induction of apoptosis has also been demonstrated both in epithelial and melanoma cells. New approaches are based on cyclooxygenase (COX)-2 inhibition by nonsteroidal anti-inflammatory drugs (NSAIDs). Diclofenac 3% gel (Solaraze™), a licensed therapy for the treatment of AK is currently being studied in organ transplant recipients (see Ulrich et al p. 40). Besides the significance of COX-2 inhibition for anti-inflammatory strategies, diclofenac 3% gel results in several anti-tumour effects, including inhibition of cell proliferation, inhibition of angiogenesis and induction of apoptosis (see Fecker et al p. 25). Although several pathways downstream of COX-2-mediated prostaglandin synthesis have been described, e.g. mitogen-activated protein kinase (MAPK) and protein kinase B activation, the pathways mediating induction of apoptosis by diclofenac 3% gel in epithelial cells, are still largely unclear and are currently under investigation.

In conclusion, suppression of apoptosis in cancer cells plays a major role in tumourigenesis, and the unravelling of the mode of activity of many anticancer agents has finally revealed apoptosis induction to be a dominant factor. First results of apoptosis-based strategies may already indicate that they are highly promising future therapies. In the thirty years since apoptosis was first described, molecular medical research has led to an enormous increase in knowledge especially in the field of apoptosis regulation. There are great hopes that there will also be therapeutic success in the near future. Importantly, the pro-apoptotic pathways exerted by specific treatments should be determined, not only to establish the effectiveness of these drugs, but also to optimise particular therapeutic approaches.

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The role of apoptosis in therapy and prophylaxis of epithelial tumours by nonsteroidal anti-inflammatory drugs (NSAIDs)

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Accepted for publication
5 March 2007

Key words
actinic keratosis, cyclo-oxygenase, diclofenac 3% gel, nonsteroidal anti-inflammatory drugs, prostaglandin E₂

Conflicts of interest
C. Ulrich has acted as a lecturer for Shire Pharmaceuticals. E. Stockfleth has acted as a lecturer/consultant for Shire Pharmaceuticals. All remaining authors declare no conflict of interest.

Summary
In addition to having anti-inflammatory activities, nonsteroidal anti-inflammatory drugs (NSAIDs) also inhibit neoplastic cell proliferation by inducing apoptosis. Diclofenac is the anti-neoplastic compound in diclofenac 3% gel (Solaraze™) used for topical treatment of actinic keratosis (AK). Main target of NSAIDs seems to be the inhibition of cyclo-oxygenase-2 (COX-2), which is overexpressed in several epithelial tumours and catalyses the synthesis of prostaglandins. The precise mechanism of action of diclofenac in cutaneous cells is still unclear, but induction of apoptosis is a key effect of anti-neoplastic drugs, including NSAIDs.

In this paper we give an overview of the anti-tumoural activities of NSAIDs with emphasis on induction of apoptosis. Cyclo-oxygenase-2-mediated synthesis of prostaglandin E₂ (PGE₂) leads to activation of mitogen-activated protein kinase (MAPK), as well as phosphatidylinositol 3-kinase (PI3K)/Akt pathways. Induction of the anti-apoptotic Bcl-2 and Mcl-1, as well as activation of the caspase-8 inhibitor cFLIP have been reported. In addition, altered lipid concentrations in the cytoplasmic membrane may modulate death receptor activities. Downregulation of both the intrinsic mitochondrial and the extrinsic pathways have been reported.

Our data demonstrate induced apoptosis and activation of the caspase cascade in three of four cutaneous squamous cell carcinoma (SCC) cell lines, after treatment with diclofenac plus hyaluronic acid and diclofenac alone; one cell line remained nonresponsive. The effects were less pronounced in normal keratinocytes and cytotoxic effects were not seen. Detailed analysis of apoptosis pathways employed by diclofenac in these cells may help to improve therapeutic strategies and to overcome possible mechanisms that are involved in non-responsiveness.

Diclofenac/hyaluronic acid used in therapy of actinic keratosis

Actinic keratoses (AKs) are epithelial tumours or carcinomas in situ derived from neoplastic keratinocytes. Their incidence correlates with ultraviolet (UV) irradiation from sunlight or artificial sources. Typically, AKs occur on the sun-exposed skin of individuals with a history of prolonged exposure to sunlight. An important group at high risk of acquiring multiple AKs and other skin tumours are organ transplant patients. Tumour genesis in this patient group seems to be related to immunosuppression, thus supporting the hypothesis of immunosurveillance.

Diclofenac is the active compound and exerts strong anti-neoplastic effects. The

Although AK neoplastic lesions are restricted to the epidermis, they may progress to invasive SCC. The risk of progression has been estimated to be as high as 8% per year, and the majority of cutaneous SCCs seems to trace back to AK precursor lesions. As there are histological similarities and molecular parallels with respect to p53 mutation, AK is now considered to be the precursor lesion of SCC. Topical therapies of AK appear to present a significant advantage over other treatment methods. Topical diclofenac 3% gel in 2.5% hyaluronic acid (diclofenac/H/A) provides physicians with an additional treatment option for AK, and its efficacy has been confirmed in clinical trials. Diclofenac is the active compound and exerts strong anti-neoplastic effects. The
mechanism of action in transformed keratinocytes remains elusive.

Functions of hyaluronic acid

Hyaluronic acid is a high molecular weight polysaccharide chain composed of a repeated disaccharide of D-glucuronic acid and N-acetylglucosamine. It is a naturally occurring extracellular matrix component. In diclofenac 3% gel, HA may help to provide a more sustained delivery of diclofenac to the skin cells. It can also bind to the receptor CD44 expressed on normal epidermal keratinocytes, which may lead to an accumulation of diclofenac and prolong its half-life in the epidermis.

Classification of NSAIDs

Diclofenac belongs to the group of NSAIDs characterised by analgesic, anti-pyretic and anti-inflammatory effects. The first active compound of this group was salicylic acid, known as aspirin. The main effect of NSAIDs seems to be the inhibition of cyclo-oxygenase (COX) enzymes, which are prostaglandin-endoperoxidases that catalyse the conversion of arachidonic acid (AA) to prostaglandins. The polyunsaturated fatty acid AA is released from membrane phospholipids as a result of phospholipase A2 activity (Fig. 1). Two human COX enzymes have been characterised; COX-1 is constitutively expressed in many cell types and COX-2, which is inducible by various stimuli, is inducible by various stimuli. A third isoform (COX-3) resulting from alternative splicing of COX-1 RNA has been found in canine cells, but owing to a frameshift mutation, it appears not to be expressed in human or rodent cells. Nonsteroidal anti-inflammatory drugs can be classified as nonselective COX-1/COX-2 inhibitors and selective COX-2 inhibitors. Diclofenac belongs to the subgroup known as arylalkanoic acids, which also includes indomethacin and sulindac (Table 1).

COX-mediated synthesis of prostaglandins

Cyclo-oxygenase activity results in the release of several prostaglandins each with different biological activities. Of these, prostaglandin E2 (PGE2) is the major mediator of inflammation as well as tumour growth. Prostaglandins are members of the eicosanoid family (oxygenated hydrophobic derivatives of C20 fatty acids) and are found in most cell types. The initial step of prostaglandin synthesis is the COX-1/2-mediated synthesis of prostaglandin H2 (PGH2) from AA, which itself gives rise to several other prostaglandins (TXA2, PGI2, PGD2, PGF2α), due to the activities of specific prostaglandin synthases (PGES). PGE2 exerts important functions in inflammation and tumour growth. Inactivation of PGE2 occurs via conversion to 15-keto PGE2 by the enzyme 15-prostaglandin dehydrogenase (PGDH).
cell surface, PGE2 can bind several receptors (EP1–EP4) of the 7-transmembrane, G protein-coupled receptor family (Fig. 2). The function of PGE2 binding to these receptors was demonstrated by homozygous deletion experiments in mice and xenotransplantation models.22–24 Prostaglandins derived from the constitutively expressed COX-1 play a role in physiological effects such as renal circulation, cytoprotection of gastric mucosa, stimulation of platelet aggregation and modulation of vascular tone.18,25 In contrast, COX-2 expression must be induced. Cyclo-oxygenase-2 is expressed in response to pro-inflammatory cytokines, growth factors and tumour promoters.26 The transcription of COX-2 is stimulated by protein kinase C (PKC), mitogen-activated protein kinase (MAPK)-mediated pathways and by NF-κB (Fig. 2).27,28 The prostaglandin synthesis resulting from COX-2 upregulation seems to be strongly related to nonphysiological conditions, such as inflammation and cancer.

**Role of COX-2-derived PGE2 in inflammation**

Cyclo-oxygenase-2-derived PGE2 is an important mediator in chronic inflammatory diseases, including arthritis and inflammatory bowel disease.29,30 In response to pro-inflammatory cytokines, such as tumour necrosis factor-α (TNF-α) or interleukin (IL)-1β, COX-2 is induced in epithelial cells, fibroblasts31 and synovial cells.32 Prostaglandin E2 is an efficient dilator of vascular smooth muscle cells and mediates vasodilatation and erythema characteristic of acute inflammation.14 The significance of COX-2 in inflammation has been clearly demonstrated in mouse models. The inflammatory response after UVB irradiation in mouse skin (oedema, dermal infiltration of leukocytes, sunburn cells) has been attributed to COX-2 activity, as demonstrated by the effects of prior topical application of selective COX-2 inhibitors (celecoxib), which reduced both PGE2 synthesis and the inflammatory response.35 Consequently, COX inhibitors are widely used anti-inflammatory drugs,36 and diclofenac has been used for the treatment of osteoarthritis, rheumatoid arthritis and acute muscle pain.37

**Anti-neoplastic effects of NSAIDs**

Several studies have demonstrated high expression of COX-2 in solid tumours of the colon,38 prostate,39 breast,40 pancreas,41 lung (non-small cell),42 bladder43 and endometrium.44 In addition, significant COX-2 expression has also been demonstrated in epithelial skin cancer. i.e. AK, basal cell carcinoma (BCC) and SCC.45,46 Furthermore, expression of the PGE2 receptor EP3 in a mouse model has been associated with tumour growth and angiogenesis.22 In addition, loss of 15-PGDH, which catalyses degradation of PGE2, has been shown to correlate with tumour formation in colorectal and lung cancer.22,47,48 The cancer-preventive and anti-tumoural activities of NSAIDs were first detected in epidemiological studies, that showed that the regular use of aspirin over longer periods of time was associated with a reduced risk for colorectal cancer.49 Regular use of NSAIDs has been further correlated with regression of pre-existing adenomas in patients with familiar adenomatous polyposis (FAP).50 However, the treatment of FAP patients with selective COX-2 inhibitors (e.g. rofecoxib; Vioxx™) had to be terminated owing to the emergence of cardiovascular adverse effects, e.g. thrombosis with this class of NSAIDs.51,52

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Fig 2. PGE2-dependent pathways related to tumour growth and apoptosis. COX-2-mediated cytosolic PGE2 may penetrate the cytoplasmic membrane by active transport through the multidrug resistance protein 4 (MRP4) and may bind to its cognate receptors (EP1–EP4) on the cell surface. Ras/Raf/MAPK and PLC/PI3K pathways seem to be activated downstream of PGE2, leading to proliferation, angiogenesis and inhibition of apoptosis. Apoptosis pathways are blocked by upregulation of Bcl-2 (mitochondrial/intrinsic pathways) and cFLIP (death receptor/extrinsic pathways). Alternatively AA-derived ceramide (Cer) may enhance DR activation. Positive feed-back loops may occur due to MAPK-mediated COX-2 induction and to activation of the EGF receptor (EGFR). Further abbreviations: PL, phospholipids; PLA2, phospholipase A1; AA, arachidonic acid; DR, death receptor; FLIP, Flice-like inhibitory protein; Csp, caspase; CM, cytoplasm membrane; VEGF, vascular endothelial growth factor; PPAR, peroxisome proliferators-activated receptor.
Diclofenac 3% gel has been shown to inhibit neoangiogenesis and to induce regression of existing blood vessels in granulomatous tissue in a mouse model. Inhibition of angiogenesis and tumour growth has also been reported in subcutaneously xenotransplanted colon adenocarcinoma tumours in syngeneic bab/l/c mice, treated with topical diclofenac 3% gel. As an indication for the mechanism of function, induced apoptosis has been reported for colon adenocarcinoma cells in vitro, when incubated with diclofenac alone. For in vivo applications, however, HA turned out as a necessary component in diclofenac 3% gel in achieving inhibitory activities on tumour growth and angiogenesis. Inhibition of tumour growth and angiogenesis, as well as induced apoptosis have also been reported for other NSAIDs, and there is a growing body of evidence indicating that these effects are related to COX-2 inhibition.

**COX-2 and tumour cell proliferation**

Cyclo-oxygenase-2 activity and COX-2-mediated PGE₂ are positive regulators of tumour cell proliferation. The effects are mediated mainly by the Ras/Raf/MAPK and PI3K/Akt-mediated pathways, which are active in a wide variety of tumours and supply major pro-proliferative signals. Expression of COX-2 itself also seems to be induced by MAPK-mediated pathways (Fig. 2). This positive feedback loop has been confirmed in intestinal adenoma and lung adenocarcinoma cells, where PGE₂ triggered enhanced cell proliferation in addition to enhanced COX-2 expression and both were induced via MAPK pathways. A further important aspect with respect to tumour cell proliferation has been reported for receptor tyrosine kinases, in particular for the epidermal growth factor (EGF) receptor, which also triggers MAPK and PI3K/Akt pathways (Fig. 2). Thus in colorectal carcinoma cells, COX-2 overexpression has been shown to stimulate cellular proliferation through induced expression of the EGF receptor. Also in colorectal carcinoma cells, combined treatment with the COX-2 inhibitor celecoxib and a monoclonal antibody blocking the Her-2/neu pathway resulted in additive growth inhibition. In addition, activation of the EGF receptor has been reported, which was dependent on the activation of Src (Fig. 2).

**COX-2 and tumour angiogenesis**

The growth of larger tumours is strongly dependant on there being sufficient blood circulation, provided by the formation of new capillaries (neo-angiogenesis), a process regulated by pro-angiogenic factors, such as vascular endothelial growth factor (VEGF). In several tumour models, a correlation between COX-2 activity and angiogenesis has been demonstrated. In human head and neck cancer, COX-2 expression correlated with tumour vascularisation, increased microvessel density and VEGF production. Also in SCC cells, an increase in VEGF mRNA and protein expression has been found in response to induction of COX-2 and enhanced PGE₂ synthesis. The COX-2 inhibitors, indomethacin and celecoxib also have been shown to reduce PGE₂ synthesis and VEGF expression in these cells.

In colon carcinoma cells held in co-culture with endothelial cells, overexpression of COX-2 has been shown to result in the formation of prostaglandins and pro-angiogenic factors, such as VEGF and basic fibroblast growth factor (bFGF). Furthermore, endothelial migration and tube formation was initiated. Using selective and nonselective NSAIDs, the production of angiogenic factors in colon cancer cells has been shown to depend on COX-2, whereas COX-1 regulated the growth and migration of endothelial cells. Further evidence for the important role of COX-2-dependent PGE₂ in VEGF production and vascularisation comes from studies using ovarian cancer cells, where the PGE₂ receptors EP1 and EP4 were found to be necessary for COX-2-mediated induction of VEGF.

**COX-2 and apoptosis**

Suppression of apoptosis (programmed cell death) is a critical feature in tumour formation. In normal cells, apoptosis leads to the ordered destruction of cellular components via several pathways. The extrinsic pathway is initiated by the binding of cell death ligands [TNF-α, TNF-related apoptosis inducing ligand (TRAIL), CD95L/FasL] to death receptors and the activation of caspase cascade. The intrinsic pathway involves activation of p53 and mitochondria, which release pro-apoptotic factors such as cytochrome c. The mitochondrial contribution to apoptosis is critically controlled by pro- and anti-apoptotic Bcl-2 proteins. Downstream of the mitochondria, caspases are also activated, and finally effector caspases cleave a large number of cell death substrates, which initiate apoptosis (for more details see the article by Eberle et al., p. 18).

Induction of apoptosis seems to be the main mode of action for the anti-neoplastic activities of NSAIDs, and in direct consequence, the anti-apoptotic potential of COX-2 is of relevance. This has been demonstrated in a number of cancer models. Strong correlations have been reported in several cancer types between COX-2 activity and the expression of anti-apoptotic proteins of the Bcl-2 family (Fig. 2). In several studies, NSAID treatment has been shown to induce apoptosis, whereas PGE₂ treatment blocked apoptosis in colon cancer cells, as well as in rat intestinal epithelial cells, which was correlated to an upregulation of Bcl-2. In addition, the up-regulation of another anti-apoptotic Bcl-2-related protein, McI-1, has been shown to correlate with COX-2 expression in BCCs. Induced apoptosis in hepatocellular carcinoma cells by NSAIDs has also been shown to be associated with a rapid down-regulation of McI-1, followed by translocation of the pro-apoptotic Bcl-2 protein, Bax, to the mitochondria and subsequent cytochrome c release.

In addition to the mitochondrial/intrinsic pathway guarded by Bcl-2 proteins, COX activity and NSAID mode of action may also affect extrinsic pathways. Apoptosis induced by sulindac sulphide, the major metabolite of sulindac, in human colon and prostate cancer cells has been shown to be accompanied by upregulation of the TRAIL receptor TRAIL-R2/DR5,
whereas levels of other death receptors were unaffected. Enhanced DR5 expression may sensitize cells to low endogenous concentrations of TRAIL and thus trigger apoptosis. For hepatocellular carcinoma cells, treatment with the selective COX-2 inhibitor celecoxib has been shown to result in upregulation of other death receptors, including CD95/Fas, TNF receptor 1 (TNF-R1) and the TRAIL receptor, TRAIL-R1/DR4.

Besides upregulation of cell surface death receptors, there is evidence of induced receptor clustering, which is a critical step in their activation. Human colon carcinoma cells were sensitised for TRAIL-induced apoptosis by DuP-697-mediated COX-2 inhibition. This was explained by an enforced clustering of TRAIL-R2/DR5 in cholesterol and ceramide-rich membrane rafts, facilitating receptor activation. Receptor clustering seemed to be initiated by the accumulation of AA as a result of COX-2 inhibition. Furthermore, high levels of AA may activate acidic sphingomyelinase, which catalyses ceramide synthesis. Ceramide has been reported to be a major force for raft formation in the cytoplasm membrane, which may be an important step in death receptor activation (Fig. 2).

A link between AA and induced apoptosis has also been reported for rat hepatoma cells. Synergistic effects have been obtained in neuroblastoma cells in response to combined treatment with AA and diclofenac. Several other effects of COX-2 activity on anti-apoptotic programs have been suggested, of particular interest is its effect on PPAR. PPAR-δ is activated by high COX-2-mediated PGE2 levels, which may be mediated through a PI3K/Akt-dependent pathway (Fig. 2). The anti-apoptotic programs mediated by the PPAR transcription factor may also contribute to the neoplastic effects of COX-2.

**Induction of apoptosis by diclofenac**

Induction of apoptosis by diclofenac has been reported in several tumour cell models. Activation of caspases has frequently been monitored. Mainly caspase-9, which is downstream of the mitochondria, together with general effector caspases were activated, whereas caspase-8 was unaffected. This was observed in neuroblastoma cells indicating a predominant involvement of the intrinsic/mitochondrial pathway.

In leukaemia cells, however, caspase-8 activation and Bid activation were found in diclofenac-induced apoptosis. This was explained as a secondary effect downstream of the generation of reactive oxygen species (ROS), as an early event in diclofenac metabolism, and downstream of inhibition of PI3 kinase and protein kinase B/Akt. The sensitivity of PI3 kinase and Akt-mediated pathways to the redox potential and their relation to apoptosis was reported in hepatoma cells.

Activation of caspase-8 after inhibition of Akt may result from downregulation of its inhibitor c-FLIP (Fig. 2). The anti-tumour effects and pro-apoptotic effects of NSAIDs have been demonstrated in vivo in xenograft models. Treatment of neuroblastoma xenografts in nude rats with diclofenac or with celecoxib significantly inhibited tumour growth in vivo. Topical treatment with diclofenac 3% gel in colon adenocarcinoma xenografts in nude mice inhibited prostaglandin synthesis and increased the apoptotic index.

**Induction of apoptosis by diclofenac 3% gel in SCC cell lines**

Topical treatment with diclofenac 3% gel is presently evolving as an efficient therapy for AK. As apoptosis appears as a key mechanism of anti-tumour therapies, we investigated the effects of diclofenac on induction of apoptosis in three cultures of normal human keratinocytes (NHK) and in four cutaneous SCC cell lines, SCL-1, SCL-II, SCC-12 and SCC-13 derived from SCC xenografts in nude mice inhibited prostaglandin synthesis and increased the apoptotic index.

The cell lines were grown in RPMI supplemented with 10% foetal calf serum, 2 mmol L⁻¹ glutamine, 1x nonessential amino acids. Normal human keratinocytes were isolated from foreskins, as described previously and were grown in CnT-02 keratinocyte growth medium (CELLTEC Advanced Cell Systems, Bern, Switzerland).

For treatment with diclofenac/HA (Shire, Cologne, Germany), cells were seeded in six-well plates (2.5 x 10⁵ cells/well) and received fresh growth medium supplemented with different amounts of diclofenac/HA (0–15%), which decreased with higher concentrations resulting in reduced apoptosis values.

As determined in several independent experiments, apoptosis was significantly induced by diclofenac/HA in three of four SCC cell lines (SCL-II, SCC-12 and SCC-13). Concentrations of 0.2–0.3% were the most effective under these conditions, corresponding to 60–90 µg diclofenac per mL, whereas higher concentrations resulted in reduced apoptosis values.

In contrast, SCL-1 failed to respond with induced apoptosis to diclofenac/HA within 24 and also within 48 h (data not shown). Rather a tendency to decreased apoptosis was seen with higher concentrations. No cytotoxicity was noted within 24 h of treatment, neither for apoptosis-sensitive cell lines nor for SCL-1 (Fig. 3a). Compared with SCC cell lines, the apoptotic response to diclofenac/HA was much lower in NHK cultures. Some apoptotic response was seen with the lowest concentration (0.15%), which decreased with higher concentrations. One of three NHK cultures was almost resistant (Fig. 4). As in the SCC cell lines, there was no cytotoxic effect in NHK within 24 h (data not shown).

**Activation of caspase-3 in response to diclofenac/HA**

To obtain further proof for diclofenac/HA-induced apoptosis in cutaneous SCC cells, activation of the main effector caspase-3 was measured by flow cytometry. The anti-apoptotic effects of diclofenac/HA on various cell lines were correlated with increased caspase-3 activity.

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spase-3 was monitored 24 h after treatment with 0.2% diclofenac/HA. Proteins were isolated from sub-confluent cultures and were analysed by western blotting (12% SDS polyacrylamide gels), as described previously. Caspase-3 cleavage products were detected by a rabbit polyclonal antibody (9661, New England Biolabs, Dreieich, Germany); equal gel loading was controlled by incubation with a β-actin antibody (Sigma Aldrich, Munich, Germany). Caspase-3 analysis clearly proved that apoptosis had been induced, as well as activation of the caspase cascade, in SCL-II, SCC-12 and SCC-13 cell lines, which had also responded in the DNA fragmentation assay. Typically, caspase-3 cleavage products of 17 and 19 kDa were detected. No caspase activation was detected in SCL-I (Fig. 3b).

Fig 3. Induced apoptosis by diclofenac/HA in SCC cell lines via caspase-3. A: Cutaneous SCC cell lines (SCL-I, SCL-II, SCC-12, SCC-13) were treated for 24 h with various concentrations of diclofenac/HA as indicated below. The apoptotic response was determined as DNA fragmentation, and cytotoxicity was determined by lactate dehydrogenase release (relative values as compared to untreated controls). Three independent experiments with at least double values were performed, and mean values ± SD of all experiments are given here. Note that apoptosis was induced best with 0.3% diclofenac/HA in three cell lines, whereas cytotoxicity was low. B: Formation of specific cleavage products of activated caspase-3 (17 and 19 kD), as determined by western blot analysis, are shown after 24 h of diclofenac/HA treatment (0.3%; +). Caspase-3 was activated in cell lines SCL-II, SCC-12 and SCC-13, whereas no activation was seen in SCL-I. For loading control, blots were incubated with a β-actin antibody.

Fig 4. Weaker apoptotic response of normal human keratinocytes to diclofenac/HA. The apoptotic response (DNA fragmentation) to various concentrations of diclofenac/HA was determined after 24 h in three independent cultures of normal human keratinocytes (NHK) and compared to SCC cell lines SCC-12 and SCC-13. Owing to very low values of untreated NHK, absolute DNA fragmentation values are given here. Mean values and SD of two or three experiments are given.
Diclofenac is responsible for the pro-apoptotic activity of diclofenac/HA

To distinguish between the effects of diclofenac and HA, cells were incubated with the individual components according to the protocols described above. Diclofenac was purchased from Sigma Aldrich. Both substances were used in equimolar concentrations equivalent to 0.2% diclofenac/HA (60 µg mL⁻¹ diclofenac and 50 µg mL⁻¹ HA, respectively). The components were applied either alone or in combination and their effects were compared with 0.2% diclofenac/HA. The effect of diclofenac (60 µg mL⁻¹) was comparable to that of 0.2% diclofenac/HA in SCL-II, SCC-12 and SCC-13. Whereas HA alone had no effect on apoptosis, its combination with diclofenac (60 µg mL⁻¹) showed a tendency to slightly increase apoptotic rates when compared with diclofenac alone. In contrast, SCL-I cells remained resistant to any treatment with diclofenac applied within 24 h (Fig. 5).

Conclusions

The NSAID diclofenac 3% gel is highly effective in the treatment of AK. However, its mechanism of activity in cutaneous neoplastic cells is still largely unclear. Induction of apoptosis in cancer cells or sensitisation to pro-apoptotic stimuli is a critical issue for anti-tumour agents, and several mechanisms have been suggested for induction of apoptosis by NSAIDs. These include both activation of the mitochondrial/intrinsic pathway, as well as the death receptor-mediated extrinsic pathways. Our data showed that diclofenac/HA induced apoptosis in three of four cutaneous SCC cell lines as shown by DNA fragmentation and caspase activation. The effects were less pronounced in normal keratinocytes, and no cytotoxic effects were seen. These data may support the hypothesis that induction of apoptosis is a key mechanism for the anti-neoplastic activities of diclofenac 3% gel in the treatment of AK.

In one of four cell lines investigated here, we observed non-responsiveness to diclofenac. A detailed analysis of the apoptosis pathways employed by diclofenac in these cells will allow a better understanding of the mechanism, how diclofenac 3% gel exerts its anti-neoplastic effects in AK, and may help to improve therapeutic strategies. Mechanisms underlying non-responsiveness to diclofenac and how they can be overcome, will be of similar importance.

References

Apoptosis by NSAIDs, L.F. Fecker et al.


Low prevalence of p53, p16\textsuperscript{INK4a} and Ha-ras tumour-specific mutations in low-graded actinic keratosis


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Accepted for publication
5 March 2007

Key words
actinic keratosis, CDKN2A, Ha-ras, non-melanoma skin cancer, p16\textsuperscript{INK4a}, p53, tumour suppressor gene

Conflict of interest
E. Stockfleth has acted as a lecturer/consultant for Shire Pharmaceuticals. All remaining authors have declared no conflict of interest.

Summary

Background Ultraviolet radiation induces DNA damage and is the major risk factor for the development of non-melanoma skin cancer (NMSC). Different mutation rates of p53, p16\textsuperscript{INK4a} and Ha-ras in cutaneous squamous cell carcinoma (SCC) and the earlier stage actinic keratosis (AK) have been reported.

Objectives To assess the presence of missense mutations in hotspot exons of p53, p16\textsuperscript{INK4a} and Ha-ras in low-graded AK.

Patients/methods Cryo-biopsies of 75 sun-exposed AK lesions and 75 sun-shielded areas of normal skin from 75 AK patients were analysed to identify mutations in p53 (exons 7 and 8), p16\textsuperscript{INK4a} (exon 2) and Ha-ras (exon 1) using polymerase chain reaction (PCR) followed by direct sequencing. As a representative subset of the specimens, ten mutation-negative AK were also micro-dissected in order to exclude the possibility that additional mutations were undetected.

Results Eight missense and one nonsense point mutations were found in the 75 AK lesions examined (12%), of which seven (9%) were tumour-specific (i.e. present in AK lesions only) and two (3%) were p16\textsuperscript{INK4a} mutations (i.e. also detected in normal skin). Three of the tumour-specific mutations (42%) were cytosine (C) to thymine (T) transitions at pyrimidine-rich sequences. Tumour-specific mutations were identified in 1% of p16\textsuperscript{INK4a} (exon 2), 1% of Ha-ras (exon 1) and at a higher rate of 7% in p53 (exons 7 and 8), including one nonsense mutation.

Conclusions The evaluation of a large number of AK specimens in this study have found a low gene mutation rate in low-graded AK lesions. p53 mutations rather than p16\textsuperscript{INK4a} and/or Ha-ras mutations may be an early event in the development of AK to cutaneous SCC.

Non-melanoma skin cancer (NMSC) is the most frequent cancer among European populations. Incidence of squamous cell carcinoma (SCC) and its early stage actinic keratosis (AK) has continued to increase over the past decade, and currently represents about 30% of all cancers. Long-term exposure of human skin to ultraviolet (UV) light is the major risk factor for the development of NMSC owing to DNA damage in epidermal cells.

Tumour suppressor genes, or more precisely, the proteins for which they encode, prevent tumour formation by exerting dampening or repressive effects on the regulation of the cell cycle. The inactivation of tumour suppressor genes resulting from DNA damage has been shown to be responsible for the onset of various cancers. Among tumour suppressor genes, p53 has been most intensely studied during the past two decades. It is organised into five structural and functional regions comprising 11 exons. Mutations of the p53 gene are considered to be early, if not initial, events in cancer development.

The precise role of p53 during skin carcinogenesis remains elusive at present. Up to 90% of cutaneous SCC specimens have been reported to contain p53 mutations which is highly indicative for skin cancer. The mutation rate in dysplastic cells is different between various exons of p53. Several hotspot regions (i.e. gene regions with a high mutation frequency) have been identified, particularly in exon 7 and exon 8. In AK lesions, the p53 mutation rate of these two exons range from 11% to as high as 48%.

The cyclin-dependent kinase inhibitor 2a (CDKN2A) uniquely encodes for two candidate tumour suppressor genes, p16\textsuperscript{INK4a} and p14\textsuperscript{ARF}. They share common exons, exons 2 and 3, but have alternatively spliced first exons: exon 1\textsubscript{a} for p16\textsuperscript{INK4a} and exon 1\textsubscript{b} for p14\textsuperscript{ARF}. Of the two tumour suppressor genes at the CDKN2A locus, p16\textsuperscript{INK4a} has been studied more intensely and is inactivated predominantly by homozygous deletion in human cancers, including NMSC. Furthermore, intragenic mutations occur in a smaller proportion of tumours and are considered rather late events during skin
cancerogenesis. Exon 2 has been identified as the hotspot region of p16\textsuperscript{INK4a} in various carcinomas but data on the mutation frequency in AK are not available at present.

In contrast to tumour suppressor genes, oncogenes are expressed in dysplastic cells and promote the development of cancer. The ras genes comprising the three p21 proteins, Ha-(Harvey)ras, N-(Neuroblastoma)ras and the two splice variants of K-(Kirsten)ras, are related guanosine triphosphate (GTP)-binding enzymes with transforming potential. Mutational activation of ras proteins promotes cancer development by interfering with a wide range of cellular processes including the regulation of cell cycle progression. More than 2000 human tumour specimens have been analysed for the presence of ras mutations, and approximately 20% of tumours are considered to harbour at least one mutation.

The identification of tumour-specific mutations in the Ha-ras gene during skin carcinogenesis of mice has generated considerable interest in studying this observation in human NMSC. Activation of the oncogene by genetic alteration takes place at the initiation, and not during progression, of tumour development. The mutational hotspot regions that are responsible for gene activation are located in codons 12 and 13 of exon 1, and in codon 61 of exon 2. Conflicting results have been reported in different studies analysing the prevalence of ras mutations in NMSC. The majority of studies have not detected any genetic alteration of Ha-ras in cutaneous SCC. In contradiction, a high frequency (46%) of guanine (G) to thymine (T) transversions in the second position of codon 12 has been found in cutaneous SCC, with at least one mutation of codons 12, 13 or 61 in 4% to 12% of cutaneous SCC specimens.

The presence of tumour-specific mutations in the p53, p16\textsuperscript{INK4a} and Ha-ras genes in early stages of cutaneous SCC, such as AK, compared with normal skin, has not been investigated comprehensively so far. AK lesions are keratotic macules, papules, or plaques with superficial scales on a red base. They should be classified as in situ SCC because keratinocytes of AK and SCC lesions are histopathologically indistinguishable without knowing the extent to which the basal membrane is involved. The degree of intra-epidermal involvement by keratinocytic atypia is graded as mild (AK 1), moderate (AK 2) and severe (AK 3). In this study, the frequency of missense mutations of p53, p16\textsuperscript{INK4a} and Ha-ras in 75 low-graded AK (comprising AK 1 and AK 2, Fig. 1) specimens, compared with normal skin tissue was analysed using polymerase chain reaction (PCR) followed by direct sequencing.

**Methods**

**Patients**

Cryo-biopsies (diameter 4 mm and a depth up to the subcutis) of 75 AK patients (aged 57–88 years old, median 71) were taken from the scalp (sun-exposed, low-graded AK lesions) and the inner site of the upper arm (sun-shielded, normal skin). All of the normal skin tissue or half of the tumour tissue samples were placed in liquid nitrogen within 2 min after resection and stored at −70 °C. The other half of each tumour biopsy was fixed in formalin and embedded in paraffin for histological evaluation. The AK lesions were defined into three grades based on histology: AK 1, AK 2 and AK 3. Grading of histological findings was performed in accordance with the histological criteria of Cockerell and Ackerman. In this study, only low-grade AK lesions (AK 1 or 2) confirmed by histology were included (Fig. 1). Ten AK specimens without mutations were also micro-dissected for validation of the results. The study was approved by the local ethics committee at the Charité, University Hospital, Berlin, Germany (number Si. 248) and was conducted according to the Declaration of Helsinki. All patients gave written consent.

**DNA isolation, PCR and sequencing**

DNA isolation of cryo-biopsies was performed using the ‘QIAamp DNA Mini Kit’ (Qiagen; Hilden, Germany) according to the manufacturer’s protocol and finally eluted in 100 μL of AE buffer (Qiagen). The micro-dissected dysplastic cells (5 μm sections of paraffin-embedded specimens) were incubated overnight in 50 μL 1x PCR buffer (10 mmol L\textsuperscript{-1} Tris, 50 mmol L\textsuperscript{-1} KCl, pH 8.3) including 2.5 μL (20 mg/mL) proteinase K at 56 °C. Proteinase K was inactivated at 95 °C for 10 min and samples were stored after centrifugation at −20 °C until further processing.

For the amplification of p53, exon 7 or exon 8, final PCR volume (50 μL) contained 1 μmol L\textsuperscript{-1} of each gene-specific primer (Table 1), 50 μmol L\textsuperscript{-1} dNTPs, 1.5 mmol L\textsuperscript{-1} MgCl\textsubscript{2} in 1 x PCR buffer (Qiagen), 0.25 units of Taq polymerase (Qiagen) and 1 μL of template DNA. The corresponding PCR

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Fig 1. Histology of a low-graded AK; hematoxylin-eosin stain: original magnification ×200. Actinic keratosis is an early stage of cutaneous SCC; both AK and SCC are stages in the development of a continuous process characterised by the proliferation of atypical keratinocytes. This figure illustrates the histology of a low-graded AK including proliferation of atypical keratinocytes that are involved in the lower portions of the epidermis, hyperchromaticity of nuclei and mitotic figures. Additionally, blue-grey fibres and homogeneity of the dermis indicate extensive solar elastosis.
was run under the following conditions: initial denaturation at 94 °C for 4 min; 40 cycles comprising the steps 94 °C for 45 s, 64 °C for 45 s, 72 °C for 45 s, and final elongation at 72 °C for 4 min. p16INK4a exon 2 was amplified using a final PCR volume (50 µL) that contained 0.5 µmol L⁻¹ of each gene-specific primer (Table 1), 250 µmol L⁻¹ dNTPs, 2.5 mmol L⁻¹ MgCl₂ in 1 x PCR buffer (Applied Biosystems, Foster City, CA, USA), 18 units of AmpliTaq Gold polymerase (Applied Biosystems), 5 µL DMSO and 1 µL of template DNA. Conditions for the PCR were: initial denaturation at 94 °C for 7 min; 35 cycles comprising the steps 94 °C for 1 min, 60 °C for 1 min, 72 °C for 2 min, and final elongation at 72 °C for 4 min. For the amplification of Ha-ras exon 1, final PCR volume (50 µL) contained 0.2 µmol L⁻¹ of each gene-specific primer (Table 1), 100 µmol L⁻¹ dNTPs, 1 mmol L⁻¹ MgCl₂ in 1 x PCR buffer (Applied Biosystems), 2.5 units of AmpliTaq Gold polymerase (Applied Biosystems) and 1 µL of genomic DNA. PCR was run under the following conditions: initial denaturation at 94 °C for 4 min; 40 cycles comprising the steps 94 °C for 45 s, 62 °C for 45 s, 72 °C for 45 s, and final elongation at 72 °C for 4 min. A second PCR using the same procedure as described above with each 1 µL of the corresponding amplicons of the first PCR was performed in order to increase the DNA amount of the genes analysed.

Each PCR product was sequenced from both directions with gene-specific primers (Table 1) and a DNA sequencing kit (Applied Biosystems) using the ABI PRISM 310 Genetic Analyser (Applied Biosystems). The sequences were aligned using the software program Se-Al v2.0a72 and compared with gene-sequences of the GeneBank entries AF307851 (p53), L27211 (p16INK4a) and NM_176795 (Ha-ras). A second independent generated PCR product was sequenced to verify detected mutations.

### Results

By direct sequencing, a total of nine (12%), including eight different, missense point mutations were found in 75 low-graded AK taken from sun-exposed sites (Table 2). All mutations were confirmed by two independent experiments. Two of these nine mutations (3%, both in p16INK4a exon 2) were also present in normal skin from sun-shielded sites. Thus, in total seven (9%) different tumour-specific mutations were detected in 75 AK 1/2 specimens. Additional mutations in p53 (exons 7 and 8), p16INK4a (exon 2), and/or Ha-ras (exon 1) were not identified in a subset of 10 micro-dissected AK specimens indicating that the number of mutations detected seems to represent all mutations present in the AK lesions of this study.

p53 exon 7, p16INK4a exon 2 and Ha-ras exon 1 harboured one (1%) single tumour-specific mutations each, and in p53 exon 8, 4 (5%) different mutation were detected. Therefore, the highest mutation rate of 7% (5/75) was found in p53 (exons 7 and 8). Single C to T nucleotide changes were most frequent and occurred in three out of seven tumour-specific mutations (43%) affecting each p53 exon 8 (amino acid change from proline to serine), p16INK4a exon 2 (amino acid change from alanine to valine) and Ha-ras exon 1 (amino acid change from threonine to isoleucine). Tandem CC to TT mutations were not found. A single adenine (A) to T mutation predicting an amino acid change from aspartic acid to valine was restricted to p53 exon 7. p53 exon 8 additionally showed single nucleotide changes each from T to C (amino acid change from glutamic acid to valine), G to A (amino acid change from aspartic acid to asparagine), and G to T (amino acid change from glutamic acid to a stop codon). In total, six missense and one nonsense tumour-specific mutations were detected, and one AK probably lost the functional p53 protein due to the nonsense mutation in exon 8 at the amino acid position 298 (Table 2).

### Discussion

Ultraviolet radiation from sunlight is known to induce DNA mutations in keratinocytes and to initiate subsequently epithelial skin cancer.8,9 In particular, UV-B fingerprint type mutations in NMSC, comprising characteristic C to T and CC to TT substitutions have been identified in the key genes p53,16,47,48 p16INK4a,25,26,49,50 and Ha-ras.51–53 However, it is only poorly understood whether the alterations of these genes are early or late events in skin carcinogenesis. In this study, we examined the mutation status of p53, p16INK4a and Ha-ras in low-graded...

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### Table 1 Specific primer sequences used for mutation analyses

<table>
<thead>
<tr>
<th>Gene/exon</th>
<th>Sequences</th>
<th>Amplicon</th>
<th>Reference</th>
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<td>P53 exon 7</td>
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<td>Exon7-F</td>
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<td>Exon7-R</td>
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<tr>
<td>P53 exon 8</td>
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<tr>
<td>Exon8-F</td>
<td>AGA GGC AAG GAA AGG TGA TA</td>
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</tr>
<tr>
<td>Exon8-R</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>P16INK4a exon 2</td>
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<td>GGG CTC AAG CTT TTC TGC TGG</td>
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</tr>
<tr>
<td>oMe2R</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Ha-ras exon 1</td>
<td>GCC AGG AGG AGA CC</td>
<td>191 bp</td>
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</tr>
<tr>
<td>HR-2-F</td>
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<td>Ex1R</td>
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AK lesions, compared with normal skin, in order to identify tumour-specific mutations. The highest mutation rate in the early stage of skin cancer was observed in the p53 gene (7%), compared with only 1% in both p16INK4a and Ha-ras.

The step-wise accumulation of genetic alterations is the crucial stage at initiation and during development of cancer. Therefore, the identification of distinct mutations, which may provide useful information as biomarkers on tumour classification, prognosis of etiopathology and response to therapy, is of prime importance in cancer research. If genetic aberrations are not repaired, or damaged cells are not eliminated by apoptosis (programmed cell death) or the immune system, consequences include cell transformation and uncontrolled proliferation. p53 is mutated in more than 50% of all human cancers including NMSC. Functions of p53 are complex and include the potential to induce apoptosis in cells with severe DNA damage, making it a key player in the protection against cancer. However, only a few studies have examined the p53 mutation rate (exons 7 and 8) from AK lesions. Taguchi and colleagues have found substitutions in three out of 27 AK lesions (11%) in those exons. In this study, a similar p53 mutation rate of 7% (5/75) was found in low-graded AK lesions. Higher mutation rates of 27% have been reported in AK. Einspahr and colleagues found p53 mutation rates of 7% in exons 7 and 8 in normal skin, and 48% in AK lesions. These discordant results may be explained in part by the different severity of the AK lesions investigated. In this study, only low-graded AK lesions were examined. Previous studies have not specified the grade of AK lesions analysed, so severe stage AK 3 lesions may have been included. Another explanation for the disparities in mutation rates observed may be the small number of samples investigated.

p16INK4a regulates the cell cycle and particularly inhibits progression through the G1 phase of the cell cycle, whereby loss of its function may lead to unrestrained cell cycling and uncontrolled cell growth; both striking hallmarks of carcinogenesis. The presence of genetic differences to reference sequences of such genes have been previously studied, although frequently without comparison to the mutational status of normal skin. p16INK4a polymorphisms have been detected, although at a low frequency, in the general population. In this study, the same A to G mutation in p16 INK4a was detected in 3% of AK lesions (2/75). However, this mutation was also detected in normal skin suggesting that this alteration is not tumour-specific and more likely a single nucleotide polymorphism (SNP) (i.e. allele frequency of 1% in the population). Moreover, this mutation may have been scored as a mutation indicating cancer if normal skin had not been investigated. Therefore, identifying sequence differences to the wild-type does not necessarily indicate tumour-specificity of mutations. Such evidence can only be obtained by comparing cancer samples with normal skin. Thus, normal skin tissue samples should be included in future mutation analyses.

So far, mutation analyses of p16INK4a have been only performed with cutaneous SCC and not with AK lesions. The mutation rate of p16INK4a (exon 2) has been shown to range between 9% and 28% in SCC. In this study, tumour-specific p16INK4a mutations were only detected in 1% of low-graded AK and consisted of a single C to T substitution.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex/age (years)</th>
<th>Histology</th>
<th>p53 (exon 7)</th>
<th>p53 (exon 8)</th>
<th>p16INK4a (exon 2)</th>
<th>Ha-ras (exon 1)</th>
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<tr>
<td>1</td>
<td>M/78</td>
<td>AK 1/2</td>
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<tr>
<td>2</td>
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<td>–</td>
<td>C50T (P278S)</td>
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<td>–</td>
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<td>3</td>
<td>M/70</td>
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<td>G59A (D281N)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>4</td>
<td>M/72</td>
<td>AK 1/2</td>
<td>–</td>
<td>T75C (E286V)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>5</td>
<td>M/67</td>
<td>AK 1/2</td>
<td>–</td>
<td>G110T (E298stop codon)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>6</td>
<td>M/75</td>
<td>AK 1/2</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>C112T (T20I)</td>
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<tr>
<td>7</td>
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<td>A292G (D125G)</td>
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</table>

Nucleotide sequences were compared to the GeneBank entries AF307851 (p53), L27211 (p16INK4a) and NM_176795 (Ha-ras). The following nomenclature was used for the mutations: the mutation with the nucleotide change at the position 104 from A to T is designated A104T and the amino acid change is shown in parentheses (e.g. change at the amino acid position 259 from D to V is designated D259V).
substitution at nucleotide position 88. Interestingly, Saridaki and colleagues found a triple CCC to TTT mutation at the same nucleotide position in cutaneous SCC. Compared with the higher mutation rate of 9–28% reported in SCC, the very low prevalence of p16 mutations in low-graded AK detected in this study suggests that alterations of this gene may occur at a later stage during the development of skin cancer.

The ras genes are the most frequently mutated oncogenes detected in human cancers, but the relevance of oncogene mutations in skin cancer development has been challenged based on a series of studies that failed to find any mutation of those genes in cutaneous SCC. In contrast, three other studies were able to detect Ha-ras mutations (exon 1) in 6% (3/50), 46% (11/24) and in 6% (2/33) of cutaneous SCC. Overall, Ha-ras mutation frequencies of 10–20% are presently assumed for epithelial skin cancer, but reports on mutations of Ha-ras in AK are scarce. Mutations of Ha-ras (exon 1) in AK lesions have been found in 4% (1/27) and 11% (2/19) of lesions. In this study, a similarly low mutation rate of 1% in low-graded AK was detected in a larger cohort. Thus, the mutation rate of Ha-ras is low in epithelial skin cancer, although it is marginally increased from AK to SCC suggesting that Ha-ras alterations are not associated with the initiation of cutaneous SCC.

In conclusion, the data from this study suggests the occurrence of p53, p16 and Ha-ras mutations are rare in low-graded AK lesions, with the highest prevalence rate of 7% in the p53 gene. Thus, p16 and Ha-ras do not seem to be involved in the early stages of epithelial skin cancer, whereas p53 is more likely to be associated with the onset of this disease.

Acknowledgments

The work of N. Krawtchenko was supported by a fellowship of the Charité, University Hospital of Berlin, Germany. This work was partially supported by a grant from the Roche Transplantation Research Foundation in Switzerland (grant number 590944305).

References

Solid organ transplantation has been performed in over one million patients worldwide and increasingly more patients are benefiting from the medical advances made in the field of transplantation. In particular, as more effective immunosuppressive therapies are developed, the overall survival-times of patients receiving transplants have almost doubled within the last 20 years.1

However, paralleling the increased survival-times of patients under chronic immunosuppression is the increased risk of cancer development. Non-melanoma skin cancers (NMSCs), especially invasive squamous cell carcinomas (SCCs), strongly outnumber all other malignancies in organ transplant recipients (OTRs). A study of renal allograft recipients found the risk of NMSC was 20 times greater compared with the general population, showing a cumulative incidence of NMSC of 7-43% at 3 years post transplantation.2 In addition, the relative risk of SCC and actinic keratoses (AKs) was found to be 100 and 250 times higher, respectively in OTRs compared with immunocompromised patients.3

In OTRs, the associated rising incidence of NMSCs, aggressive behaviour of NMSCs (i.e. metastasising rate: 6–9%) and high mortality rates (i.e. 50%) can be explained mainly by an impaired cutaneous immuno-surveillance that allows dysplastic keratinocytes to proliferate and spread at an accelerated rate.4 Moreover, the large numbers of invasive SCC that accumulate from earlier forms of dysplasia in sun-exposed areas warrant adequate tools for the comprehensive clearance of early, subclinical and advanced forms of dysplasia (i.e. AKs) within these ‘dysplastic fields’ in order to prevent further morbidity and mortality.5

Diclofenac is a non-steroidal anti-inflammatory drug (NSAID) that is thought to exert its actions by inhibiting the
inducible cyclo-oxygenase 2 (COX-2) enzyme and reducing the production of prostaglandins. Sun-damage, AKs and invasive SCC have been linked with increasing levels of prostaglandins and COX-2 activity, paralleling increased levels of dysplasia.\textsuperscript{6} Diclofenac has been shown to inhibit murine angiogenesis and has a significant anti-tumour effect in murine colon-26 growth.\textsuperscript{7} Furthermore, diclofenac may act via an over expression of metalloproteinases, which have keratolytic and collagenolytic effects.\textsuperscript{8}

Topical diclofenac gel is formulated in 2.5% hyaluronic acid (HA), a natural high molecular weight linear polysaccharide. Hyaluronic acid is found in the extracellular matrix of most mammals, including the skin in order to stabilize and to protect the connective tissue. Hyaluronic acid enhances the delivery of drugs to designated sites, especially to areas of pathology where there is a high expression of hyaluronan receptors, such as CD44 and intercellular adhesion molecule-1. These receptors are upregulated at inflammatory sites.

Several randomised, double blind, placebo-controlled studies using topical diclofenac 3% gel (Solaraze\textsuperscript{TM}; in 2.5% HA) for the treatment of AKs in immunocompetent patients have shown complete clearance rates of between 30–50%.\textsuperscript{9–11} In addition, a recent bilateral comparison study between diclofenac 3% gel and 5-fluorouracil (5-FU) showed comparable efficacy for both treatments (i.e. clearance rates of 89% and 98%, respectively).\textsuperscript{12} Diclofenac 3% gel was associated with only mild signs of inflammation compared with 5-FU, despite a longer treatment period (i.e. 90 days compared with 28 days, respectively).\textsuperscript{12} Thus, evidence exists to suggest a potential beneficial use of diclofenac 3% gel in the treatment of AKs in OTRs. This study is the first study designed to investigate the effect of diclofenac 3% gel on clearance rates of multiple AKs in OTRs.

**Methods**

In this open-labelled, uncontrolled, non-randomised study, six OTRs (three kidney, one liver and two heart transplant patients) with histories of multiple SCC, BCC, Bowen’s disease and extensive AKs were enrolled. Patients with more than or equal to three lesions on either the forehead, central face or scalp were treated with diclofenac 3% gel, applied twice daily for 16 weeks. Patients were advised to wash the treatment area with mild soap and water and allow it to dry thoroughly before applying the treatment gel. Patients were also encouraged to leave the treatment gel on for approximately 8 h after each application. The size of the treatment area was 50 cm\textsuperscript{2}. In total, each area contained between four and 10 clinically evident AKs. In each patient, punch biopsies (4-mm in diameter) were performed centrally in one of the clinically identified AKs within each treatment area. The first biopsy was taken at the beginning of the study before the first application of diclofenac 3% gel and another at 4 weeks after the end of treatment. Complete (100%) and partial clearance of AKs (\geq 75%) were assessed clinically 4 weeks after end of treatment and confirmed histologically at Week-20 (i.e. 4 weeks at end of treatment).

All consenting patients were over 18 years of age with stable immunosuppression during the previous six months prior to study enrolment. In addition, all patients remained on the same immunosuppressive therapy throughout the study period. Laboratory parameters were routinely checked by the transplant centre and special attention was paid to serum creatinine levels.

**Results**

This study demonstrated that in three out of six patients treated with diclofenac 3% gel, all AK lesions were clinically and histologically cleared (i.e. 50% of patients had achieved complete clearance. Figs 1 and 2 show the effect of treatment with diclofenac 3% gel on AK lesions. Two patients (33%) obtained a partial clearance (i.e. \geq 75% clearance of all AKs in the treated area) and one patient (17%) achieved 30% lesion reduc-

![Fig 1. AK lesions on the nose prior to treatment with diclofenac 3% gel.](image1)

![Fig 2. AK lesions on the nose following treatment with diclofenac 3% gel.](image2)
tion after 16 weeks of treatment with diclofenac 3% gel (Fig. 3).

No wound infection or scarring was observed in any of these patients. Adverse effects were mild and consisted mainly of erythema and marginal erosion at the site of application. In addition, there were no reports of any systemic side effects. Creatinine levels remained stable during the treatment period and no effect on systemic immunity or on the graft was observed.

Discussion

The results of this preliminary study demonstrate that diclofenac 3% gel is beneficial for the local treatment of AKs in OTRs. The accelerated skin carcinogenesis seen in immunocompromised OTRs makes them the ideal population to study short term efficacy rates in the clearance of AKs and long term prevention of invasive SCC. The results of this study is in keeping with the findings of other trials that show the benefits of the so-called ‘field or topical therapies’ compared with keeping with the findings of other trials that show the benefits of the so-called ‘field or topical therapies’ compared with non-topical therapies (e.g. cryotherapy) that are unspecific.

Evidence exists to suggest that the transplant population would benefit from a self-applied effective and safe method to treat clinical and subclinical AKs. A larger, placebo-controlled, randomised trial on diclofenac 3% gel for the treatment of AKs in OTRs is due for completion with published data available early in the year.

The management of skin diseases in OTRs remains an exciting and interesting area, providing rewarding opportunities for dermatologists within the field of transplant medicine. In addition, the findings of this study are also likely to have significance for disease-related (i.e. HIV), therapeutically induced immunosuppression and autoimmune-disorders, including multiple dermatological conditions requiring topical or systemic immunosuppression where high levels of NMSC may be seen.

References

Management of actinic cheilitis using diclofenac 3% gel: a report of six cases

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Accepted for publication
5 March 2007

Key words
actinic cheilitis, COX-2 inhibitor, diclofenac 3% gel, field management, squamous cell carcinoma

Conflict of interest
C. Ulrich has acted as lecturer for Shire Pharmaceuticals. E. Stockfleth has acted as a lecturer/consultant for Shire Pharmaceuticals. All remaining authors declare no conflict of interest.

Summary
Background Actinic cheilitis is a frequent manifestation of actinic dysplasia and requires early therapy to prevent its progression into invasive squamous cell carcinoma (SCC). Several therapies are used, ranging from unspecific lesion-adapted destructive techniques (i.e. laser) to ambitious surgical field-management (vermillionectomy). There is increasing awareness of the effectiveness of field adapted, non destructive therapies, such as photodynamic therapy or 5% imiquimod. Diclofenac 3% gel is used in the treatment of actinic keratosis (AK), but it has not been evaluated for the treatment of actinic cheilitis.

Objectives This non-blinded, uncontrolled case series study evaluated the effects of diclofenac 3% gel in the treatment of actinic cheilitis.

Patients/methods Six patients with histologically verified actinic cheilitis were treated with diclofenac 3% gel, twice daily for 6 weeks. Clinical assessment was performed 2–4 weeks after the end of treatment.

Results Four out of six patients showed clinical clearing of actinic cheilitis 2–4 weeks after the end of treatment. Biopsies were taken from the treated areas at the final visit to verify clinical clearance. Side effects in most of the patients included mild erythema and mild to moderate swelling of the lips.

Conclusions Topical therapy with diclofenac 3% gel may be an efficient, cosmetically more appealing alternative treatment for actinic cheilitis than currently used destructive therapies. However, future studies and long-term follow-up of patients will be needed to compare its efficacy with established forms of therapy.

Actinic cheilitis is the analogue of actinic keratosis (AK) and is often seen in the same patients. In addition to smoking, long-time exposure to ultraviolet (UV) light represent the main risk factor for its development. Actinic cheilitis mostly presents on the lower lip. As with AK, actinic cheilitis has the potential to progress to invasive squamous cell carcinoma (SCC). Currently, there is insufficient data relating to progression rates. However, with a reported incidence of 1.8 per 100 000 cases per year, SCC of the lip is the most common oral cancer. In a study of 65 patients with actinic cheilitis presenting to the department of oral medicine in Thessaloniki, Greece, 11 (16.9%) had histological signs of invasive SCC.

The clinical picture of actinic cheilitis ranges from mild erythema, and localised or diffuse, persisting, whitish, papillary scalyness, to atrophy with focal hyperkeratosis and recurrent erosions. Since invasive SCCs of the lip have an increased risk of metastasis, early treatment of actinic cheilitis is obligatory. As with AK, surgical and non-surgical treatments are available (Table 1). However, surgical excision (vermillionectomy) followed by laser treatment (carbon dioxide), cryotherapy and electrodissection are most frequently used, and have previously shown good clinical effectiveness. Histological findings have confirmed lesion clearance in CO2 laser-treated patients, and there is less scarring than after vermillionectomy. Interestingly, in this study 5-fluorouracil (5-FU) failed to achieve complete removal of histologically proven dysplasia. Favourable results with topical imiquimod 5% in treating actinic cheilitis have been reported by Smith et al. 2002, although aphthous-like ulcerations have been associated with this treatment.

Diclofenac 3% gel (Solaraze™ 3% gel) is currently licensed for the treatment of AK in Europe and the US. Recent studies have shown that the clinical efficacy of diclofenac 3% gel is comparable with topical 5-FU and that it is favoured by most patients because it has a lower rate of side effects. In this non-blinded, uncontrolled case series study, the efficacy of diclofenac 3% gel for the treatment of actinic cheilitis was evaluated.
Patients and methods

Six patients with histologically confirmed actinic cheilitis were treated with diclofenac 3% gel (Table 2). Five patients showed significant actinic dysplasia at the typical site of the lower lip, while one patient had more profound damage on the upper lip. A 3–4 millimetre punch biopsy was taken from the clinically affected skin to exclude an invasive SCC. Patients applied diclofenac 3% gel twice a day for a total period of 6 weeks. All patients developed swelling of the lip after the first 1–2 weeks of treatment. Consequently, in two cases the application was reduced to every other day. To confirm the clinical outcomes additional biopsies were taken at Week 6.

Results

All patients finished the 6 week treatment course. All patients developed painless swelling of their lip after the first 2 weeks of treatment. No erosion or ulceration was seen. Following discontinuation of treatment, the swelling resolved after an average period of 2 weeks. Treatment was continued until the end of Week 6, at which time four out of six patients presented with no clinical signs of actinic dysplasia of the lip. Biopsies taken at Week 6 confirmed that four out of six patients experienced a total clearance of actinic cheilitis. Thus, there was 100% correlation between the clinical outcome and the histological findings. The remaining two patients had a partial response to treatment with diclofenac 3% gel. They showed partial clinical improvement but still had detectable signs of actinic dysplasia. There was no wound infection or scarring observed in any of the six patients. The cosmetic outcome was excellent.

Discussion

These case studies show for the first time that diclofenac 3% gel may be a well tolerated, effective therapeutic option for
the treatment of actinic cheilitis. Diclofenac 3% gel is currently licensed in Europe and the USA for the treatment of AK, and it has also been shown to be a successful therapy of Bowen’s diseases. In a meta-analyses of three studies with a total of 364 patients with AK, diclofenac 3% gel produced complete resolution of lesions in 40% of cases and a partial response in 75%. To achieve this, a treatment duration of 60–90 days was required.

The exact mode of action of diclofenac 3% gel is not well understood. At first glance treatment of pre-malignancies with diclofenac, a nonsteroidal anti-inflammatory drug (NSAID) appears contradictory. It inhibits both cyclo-oxygenase (COX)-1 and 2 (COX-2 more than COX-1), thereby blocking the initial step in the formation of prostaglandins in a variety of pathophysiological processes. But beyond its analgesic properties, COX-2 is up-regulated in a number of malignancies including carcinomas of the lung, bladder and oesophagus, and has recently been described as a new target for anti-cancer drug development. However, in all these studies COX-2 inhibitors alone failed as a tumour treatment, but showed a promising adjuvant effect in combination with chemotherapy.

Studies using human cell lines show a causal link between the up-regulation of COX-2 and increased levels of the anti-apoptotic protein, Bcl-2. The level of COX-2 also correlates with vascular endothelial growth factor (VEGF) expression and the subsequent vascularisation of cancers. The importance of VEGF in the development of SCC of the lips has been previously documented. Interestingly an up-regulation of COX-2 has also been described in early or advanced SCCs including AK. Cyclo-oxygenase-2 over-expression in squamous cell head and neck cancer has also been previously demonstrated. In contrast however, non-sun-exposed control skin and basal cell carcinoma (BCC), despite being integrated in the family of NSMC, have shown no expression of COX-2 in immunohistochemical stainings and western blots.

One major finding of our study is that the clinical and histological response induced by diclofenac 3% gel correlates with erythema and swelling of the lip. The skin barrier appears to be an important factor for determining therapeutic outcome. Despite the obvious limitation of having a small number of treated patients in this study, complete healing of the actinic cheilitis lesions occurred in 66% of cases, compared with only 40% in AK. The thicker, hyperkeratotic skin of the scalp may be responsible for the significantly lower response rate seen in AK and the longer treatment duration needed. We accept that inflammation is a prerequisite for successful treatment with diclofenac 3% gel. Indeed, treatment is usually terminated if there is no inflammatory response around the actinic lesions within 4 weeks.

So how can an anti-inflammatory COX-2 inhibitor induce an inflammatory immune response? Our clinical findings are comparable to the treatment of BCC with 5% imiquimod. Imiquimod binds to Toll-like receptor 7 (TLR-7) on dendritic cells within the epidermis and induces a T-cell driven inflammatory response, which destroys the tumour cells within 4–6 weeks of treatment. With diclofenac 3% gel, hyaluronic acid (HA) contained in the preparation may be of importance. Hyaluronic acid is an ubiquitous, extracellular matrix component, present at high concentrations in the skin. In normal, unaffected skin, HA exists as a high molecular weight form, and has a function in the maintenance and hydration of the cutaneous extracellular matrix. However, at sites of inflammation or bacterial infection, HA undergoes rapid degradation.

The resulting low molecular weight degradation products of HA have been found to elicit various pro-inflammatory responses, such as the activation of alveolar macrophages, as well as dendritic cells of the skin. Similar to imiquimod the activation is driven by the Toll-like receptors, TLR-2 and TLR-4. It has been suggested that low-molecular-weight hyaluronan species act as an endogenous danger signal and both forms of HA together work as a tissue repair system.

Despite the complete absence of data on the immunological effects of diclofenac 3% gel, it may well be that the gel is degraded by hyaluronidase containing Streptococci that colonise the hyperkeratotic actinic lesions. This in turn induces the inflammatory response that was observed in this study and simultaneously releases the diclofenac from the gel that acts as an adjuvant for the elimination of the tumour cells.

Conclusions

To date, multiple studies have shown that diclofenac 3% gel is an effective and well tolerated field therapy for AK. Although there was a low sample number and a short follow-up period, data from this study suggests that diclofenac 3% gel may have a place in the treatment of actinic cheilitis. Despite the advantages of this effective, cosmetically well acceptable and non-invasive therapy, biopsy is still indicated for lesions that have developed substantial thickness, induration of the base, or ulceration, in order to exclude initial or advance invasiveness and the potential risk of metastasis in these high-risk patients. In addition, further studies are needed to establish optimized treatment algorithms for the management of actinic cheilitis with diclofenac 3% gel. More convenient forms of diclofenac 3% gel could be developed, e.g. a lipstick formulation that can be applied onto the lips, which may be used to treat fully prevalent actinic dysplasia, as well as being a prophylactic treatment for at-risk patients.

References

Managing actinic cheilitis with diclofenac 3% gel, C. Ulrich et al.


Differentiation between actinic keratoses and disseminated superficial actinic porokeratoses with reflectance confocal microscopy

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Summary

Background Clinical differentiation between actinic keratosis (AK) and disseminated superficial actinic porokeratosis (DSAP) may pose a significant challenge, and histological evaluation is often also required for diagnosis. Distinct morphological features can be distinguished upon histopathological examination, but the use of non-invasive tools, such as reflectance confocal microscopy (RCM), may be an eligible alternative for confirmation of diagnosis.

Objectives The aim of this study was to determine the relevant RCM criteria for the identification of disseminated superficial actinic porokeratoses (DSAPs) and to define distinguishing criteria for DSAPs compared with actinic keratosis (AKs).

Patients/methods A total of 20 patients with a clinical diagnosis of AK or DSAP were included in this study. All lesions were evaluated by clinical examination, and RCM and one clinically identified lesion was biopsied for histological confirmation.

Results Cellular and nuclear atypia, inflammation, spongiosis, parakeratosis and changes in epidermal architecture were present in both lesion types (i.e. AKs and DSAPs). However, these features were more pronounced in AKs. Whereas AKs exhibited more disseminated parakeratotic changes, parakeratosis was found focally present on the border of DSAP lesions. Most characteristically, a distinct border corresponding to cornoid lamella in RCM can be identified in DSAPs.

Conclusions Distinguishing features of DSAPs, such as cornoid lamella, sharp demarcation of the lesion and focal keratinocyte atypia are easily identifiable using RCM, and correlate well with histopathology. Whilst RCM has previously been used in the evaluation of AKs, it has not yet been used to investigate DSAPs. The findings in this study suggest the feasibility of non-invasive tools, such as RCM for the differentiation of AKs and DSAPs. However, further studies are warranted to assess the sensitivity and specificity of RCM in the diagnosis of DSAP.

Disseminated superficial actinic porokeratosis (DSAP) presents clinically as erythematous or brown, flat papules or plaques with a slightly raised keratotic border and a slightly atrophic centre. Disseminated superficial actinic porokeratosis is a keratinisation disorder of unknown aetiology, affecting women and men in their third decade of life. Sun exposure and immunosuppression are well established risk factors and an autosomal dominant mode of inheritance with reduced penetrance has been described for DSAP. Disseminated superficial actinic porokeratoses (DSAPs) frequently occur on sun-exposed areas of upper and lower extremities, but may also affect the face. Malignant transformation of DSAPs to squamous cell carcinomas (SCCs) is relatively rare; however a progression rate of 3.4% has been reported.1

Actinic keratosis (AK) have been defined as squamous cell carcinoma (SCC) in situ.2,3 Actinic keratoses (AKs) occur on chronic sun-exposed areas and usually present as erythematous, keratotic plaques. Ultraviolet light is the main risk factor and fair skin type, out-door occupation and longstanding immunosuppression contribute to the development of AKs. Elderly people above the age of 60 are commonly affected, but changes in recreational behaviour in the past decade with
frequent sunbathing and use of tanning beds have led to an increased incidence of AK in the younger population. Progression of AKs to invasive SCC has been reported in up to 20% of cases with immunosuppressed patients being at greater risk.  

Both lesion types (DSAPs and AKs) have several clinical features in common, i.e. they may present as erythematous, keratotic plaques and occur on sun-exposed areas. Upon clinical evaluation, DSAPs show a distinct, elevated margin, often allowing the distinction from AKs to be made. However, for a clearer diagnosis, biopsies may be required.

Upon histopathological examination, DSAPs characteristically show a cornoid lamella, representing a circumscribed column of parakeratosis at the lateral margin of the lesion. Underneath the cornoid lamella the granular cell layer is absent, and the epidermal cells show atypia and irregular arrangement. A slight lymphocytic infiltrate and dilatation of blood vessels may be seen in the upper dermis.

Actinic keratoses are characterised histopathologically by parakeratosis, keratinocyte atypia, cellular and nuclear polymorphism, increased rate of mitosis and loss of epidermal architecture. Usually an inflammatory infiltrate, mostly composed of lymphocytes, is found in the upper dermis underlying the neoplastic changes. Several histological types of AKs have been defined histologically, including hypertrophic, atrophic, lichenoid, bowenoid, pigmented and ancantholytic variants.

Reflectance confocal microscopy (RCM) is a high-resolution technique, which allows the non-invasive evaluation and diagnosis of normal and diseased skin lesions in vivo. It has been used in a number of dermatological clinics for the evaluation of a variety of skin neoplasms, such as basal cell carcinoma (BCC), AKs and malignant melanoma.  

Materials and methods

Subjects

A total of 20 patients with skin phototypes I, II and III participated in the study. Written consent was obtained from each participant prior to the study. Ten patients had histologically confirmed AKs and the other 10 had DSAPs. A history check was performed in each of the patients, followed by clinical examination and digital photography of their lesions. Patients with a history of other skin diseases and hyperkeratotic AKs were excluded from the study. All skin sites were evaluated by clinical examination, RCM and routine Haematoxylin and Eosin (H&E) histology.

RCM imaging

Confocal imaging was carried out using a commercially available microscope (Vivascope 1500, MAVIG, Munich, Germany) equipped with an 830 nm diode laser. The 30× objective lens with a numerical aperture of 0.9 was applied to the skin by using ultrasound gel as an immersion medium. Further technical details of this system have previously been published. One lesion clinically suspicious for either AK or DSAP was selected for imaging in each patient. Systematic horizontal
mapping of every lesion was performed where 4–6 images of 500 × 500 µm were captured, each beginning in axial sections from the stratum corneum to the stratum spinosum, granulosum and into the upper dermis.

For the diagnosis of AK, parameters have been based on previously published criteria and all sites were systematically evaluated for the presence or absence of RCM features indicative of DSAP and AK by two experts in the field of RCM (see Table 1). As RCM has not previously been used for the evaluation of DSAPs, the evaluation criteria were based on those established by routine histology and included parakeratosis, distinct margin in the periphery (cornoid lamella), cellular atypia, disrupted epidermal architecture, blood vessel dilatation and inflammatory infiltrate. Reflectance confocal microscopy (RCM) images were then individually subjected to evaluation by two independent observers for final scoring.

### Routine histology

All patients underwent punch biopsy, performed at the same site of RCM imaging, in order to confirm the diagnosis. The sections were fixed in formalin, embedded in paraffin and 4 µm sections were stained with H&E. Blinded evaluation of all specimens was performed by a board certified dermatopathologist.

### Results

#### Actinic keratoses

Upon RCM evaluation, AKs showed the presence of severe, circumferential parakeratosis, separation of individual corneocytes and commonly impetiginisation at the level of the stratum corneum. Within the granular and spinous cell layer, architectural disarray was seen and atypical keratinocytes with cellular and nuclear polymorphism were found. Furthermore, inflammatory cells in the epidermis (exocytosis) and spongiosis were commonly observed. Superficial dermal features included dilatation of blood vessels and clumping of fibres in the papillary dermis representing solar elastosis. A sharp border surrounding the AK lesions could not be observed, as signs of actinic damage were present in the periphery of the lesions. Reflectance confocal microscopic features of AKs were correlated to routine histology and were consistent with previously published findings of AKs. Common characteristic features of AKs are as illustrated in Fig. 2.

### Disseminated superficial actinic porokeratoses

Disseminated superficial actinic porokeratoses showed several similarities with AKs upon RCM evaluation, which include parakeratosis, individual corneocytes, architectural disarray, cellular and nuclear atypia, spongiosis and dilatation of blood vessels. One distinguishing factor was that all DSAP lesions showed a distinct, well demarcated and raised border to the normal skin, which corresponded to the presence of cornoid lamella in routine histology. In addition, loss of epidermal architecture and atypia were generally less severe in DSAPs than in AKs, and solar elastosis was not commonly present in DSAPs. Reflectance confocal microscopy findings of DSAP were confirmed by routine histology. Compared with the RCM findings of AKs, DSAPs showed a sharp border and had normal skin surrounding the lesion. Common characteristic features of DSAPs are shown in Fig. 3.

### Discussion

Lesions from DSAP patients may clinically resemble those of AKs and so currently the gold standard for the diagnosis of DSAP is histopathological examination. Upon histopathological examination of DSAPs, keratinocyte atypia and irregular epidermal architecture can be observed. Most characteristically, DSAP lesions show a cornoid lamella, an area of localized parakeratosis on histopathology, which underneath show a loss of the granular layer. In order to find the cornoid lamella, a biopsy has to be taken at the margin of the lesion. Nevertheless, the diagnosis may pose significant difficulties for the pathologist. Correct diagnosis remains crucial for the therapeutic management of both DSAP and AK patients. Notably, AKs have been defined as carcinoma in situ with a reported progression rate to SCC ranging from 10 to 20%. Actinic keratoses occur on chronically sun-exposed skin and atypical cells are often detected in the area surrounding the lesions, which lead to the concept of \"field cancerisation.\" Therefore effective treatment of all lesions is important and field therapies have been developed for the management of AK.

Disseminated superficial actinic porokeratoses are less common than AKs and occur on sun-exposed areas, mainly on arms and legs. Progression of DSAPs to SCCs has been described, but seems to be less common than in AK or other forms of porokeratoses. In vivo RCM has been shown to be a useful tool for the evaluation of a variety of neoplastic and inflammatory skin conditions. Reflectance confocal microscopy

| Table 1 Reflectance confocal microscopy features of DSAPs compared with AKs |
|---------------------------------|----------------|----------------|
| **Dermal level**                | **DSAP** | **AK** |
| Epidermal atypia                | +        | +        |
| Architectural disarray          | +        | +        |
| Cellular and nuclear atypia     | +        | +        |
| Inflammation/exocytosis         | +        | +        |
| **Dermal level**                |          |          |
| Solar elastosis                 | –        | +        |
| Blood vessel dilatation         | +        | +        |

Illustrates the presence (+) and absence (−) of selected histomorphological features for DSAPs and AKs as evaluated by RCM.
allows non-invasive examination of multiple skin sites and repeated imaging of a selected lesion over a period of time. In this study, the RCM features of DSAP and AK lesions, in correlation with routine histology, were evaluated. At the level of the stratum corneum, parakeratosis, individual corneocytes and impetiginisation were present in both DSAPs and AKs. However, parakeratosis in DSAPs presented with rather focal changes compared with AKs. Changes of the stratum granulosum and spinosum include cellular and nuclear atypia, architectural disarray, spongiosis and inflammation. At the level of the superficial dermis, dilatation of blood vessels was observed in DSAPs and AKs. Atypia, loss of normal epidermal architecture and inflammation appeared more severe in AKs. In terms of distinct patterns, marked solar elastosis as an indicator of chronic sun damage was usually present in AKs, but were only subtly present in DSAP. The most characteristic finding for the differentiation of DSAPs and AKs was the distinct lateral margin of the DSAP lesion, which correlated with the presence of cornoid lamella in routine histology.

In summary, some of the described features were present in both AKs and DSAPs, but were generally more pronounced in AKs. The presence of a cornoid lamella and well defined demarcation appears to be a diagnostic feature for DSAP, as these findings were usually absent in AKs. The preliminary findings herein suggest the applicability of RCM for the differential diagnosis of AKs and DSAPs. However, some differences were subtle and larger studies are warranted to evaluate the validity, sensitivity and specificity of our findings.

Fig 2. Common characteristic features of AKs. Representative RCM images of DSAP in horizontal sections. Panels (a)–(c); shows changes of the stratum corneum with disarray (a), parakeratosis (b) and individual corneocytes (c). Panels (d)–(f); illustrates architectural disarray (d), with atypical keratinocytes (e) and spongiosis (f) of the granular and spinous cell layer. Panel (g) shows dilated capillaries in the upper dermis with increased tortuosity of blood vessels. Panel (h) illustrates inflammatory cells (white arrowheads) and (i) shows the distinct margin in the periphery of a DSAP lesion corresponding to the cornoid lamella. RCM image 500×500 microns.
findings. Furthermore, a histomorphologic differentiation between epidermal lesions (i.e. DSAPs, AKs) and invasive SCC was not possible as the penetration depth of RCM is limited to the epidermal and upper dermal layer. This study was also limited by the small number of subjects included in this investigation, which further substantiates the need for larger studies in order to demonstrate the clinical applicability of these findings.

Acknowledgments

This study was supported by departmental funds and the authors would also like to acknowledge MAVIG GmbH for providing the Vivascope 1500 for this study.

References

Does progression from actinic keratosis and Bowen’s disease end with treatment: diclofenac 3% gel, an old drug in a new environment?

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Summary

Progression from actinic keratosis (AK) and Bowen’s disease (BD) to invasive disease involves a complex cascade of events. The preparation of diclofenac 3% gel (Solaraze™; Shire Pharmaceuticals) has been shown to be efficacious and well tolerated in AK. The inhibition of the COX enzymes results in a decrease in downstream by-products of arachidonic acid metabolism. These metabolites have been shown to play a pivotal role in promoting epithelial tumour growth. Given its mechanism of action, we hypothesize that diclofenac 3% gel may have potential to halt the progression of actinic keratoses (AKs) in the setting of field cancerisation and BD. We report a series of five patients with BD, all treated with diclofenac 3% gel with clinical and histological clearance.

Non-melanoma skin cancer (NMSC) is the most common cancer in the UK, and it has been reported that over a 10 year period, the incidence of NMSC in the UK has increased from 174 to 265/10^5/year.1 The incidence of NMSC continues to increase in Europe and throughout the world. The two most frequent manifestations of invasive NMSC are basal cell carcinoma (BCC) and squamous cell carcinoma (SCC). There is, however, an important biological difference between these two types of NMSC, which is that progression to invasive SCC is often first characterised by the development of actinic keratosis (AK) or Bowen’s disease (BD).

Actinic keratosis typically develops on areas of the skin exposed to the sun, and commonly presents as red, scaling papules or plaques. However, this may be the only visible manifestation of a larger area of ‘field cancerisation’, in which subclinical lesions also play an important role. If any of the lesions in this AK field are incompletely removed there is a strong chance of reoccurrence, and if left untreated there is the potential to develop into invasive SCC, which may metastasise to other structures or internal organs. Although early visible lesions may be only a few millimetres in size, the AK field may extend to several centimetres in diameter. Even if AK lesions clinically regress they can reoccur at a later date.

Actinic keratosis is very common with as many as one in six people developing actinic keratoses (AKs) during their lifetime. More than 34% of men over the age of 70 have at least one AK lesion,2 and it is estimated that up to 10% of AK lesions progress to SCC annually.3 Normally however, multiple rather than single AKs occur. Although these lesions tend to be harmless initially, it is important to recognise and acknowledge that AK is more than simply a cosmetic skin problem. AK is a carcinoma in situ and is the first stage of a disease continuum that can invariably progress from subclinical lesions to invasive SCC. Consequently, AK must be diagnosed correctly and treated promptly.

Development of AK and progression to full-thickness and invasive SCC

Actinic keratosis is a consequence of chronic sun-damage to the skin. Not surprisingly, it is most common in fair-skinned individuals with red or blond hair, who burn easily in the sun and tan poorly.4 Thus, the principle risk factors are cumulative exposure to ultraviolet (UV) light and skin phenotype (AK is more prevalent in skin types I and II). Individuals whose immune systems are compromised as a result of cancer chemotherapy, AIDS or organ transplantation are also at greater risk.

Actinic keratosis rarely presents as a single lesion. It is often found in an area of skin that has undergone extensive field damage (field cancerisation),5 in which a cascade of tumour progression occurs at different stages at multiple sites throughout the sun-exposed skin, starting with subclinical lesions leading to early AK, late AK, and eventually to invasive SCC. Within the AK field, therefore, skin tissue undergoes a series of subtle biological and genetic changes. Actinic keratoses form part of a continuum that begins with DNA damage and mutation, neoplastic transformation and proliferation of
keratinocytes. These transformed cells do not mature normally. Depending on many factors, particularly the host’s immune response and the background normal keratinocytes, these lesions may remain stable, or enlarge and extend into the dermis.6

Progression of AK to full thickness atypia and invasion involves a complex cascade of events including signal transduction, cell proliferation and inflammation. A number of specific mechanisms are known to play a key role, including gene mutation, reduced apoptosis (programmed cell death), increased cell proliferation, modification of the ras oncogene, magnified cyclooxygenase (COX) expression and diminished immune response. (See Eberle et al., p. 18).

Gene expression profiling during skin carcinogenesis has revealed the complexity of the underlying pathophysiology of AK, and how it is possible to immobilise the process that normally prevents progression to malignant disease. In normal skin, DNA damage leads to the stimulation of repair mechanisms, such as the presence of tumour suppressor genes (e.g. p53 and p16), and induction of apoptosis. However, UV radiation can cause the p53 gene to mutate, acting as a catalyst in the initiation of the AK/SCC cascade. Skin biopsies show that early, mild AK lesions (stage I) harbour p53 gene mutations (stage I: p53).7 A comparative study of SCC and AK human samples by loss of heterozygosity (LOH) analysis determined that the p16 (INK4a/ARF) locus is less frequently altered in AKs than in SCCs. These results implied that progression of AK into SCC may involve inactivation of p16 (INK4a).8 Further studies are necessary to better understand the role of gene mutations in the progression from AK to SCC.

The increased expression of COX-2 has been shown in AK, BD and SCC.9 It is also known that the COX-2 pathway plays an important role in the AK/SCC cascade, and that by inhibiting this pathway it may be possible to prevent the progression of early AK lesions to invasive SCC, and clear existing AK lesions.10,11 The role of the immune system in the development of AK is highlighted by the increased susceptibility to skin cancer in organ transplant patients. Within the first 5 years of immunosuppression 40% of transplant recipients develop skin tumours including AK, BD, invasive SCC and BCC.12 In an Australian population, the cumulative incidence of developing skin cancer increased progressively from 7% after 1 year of immunosuppression to 45% after 11 years and to 70% after 20 years.13

This complex cascade of genetic mutation, tumour genesis and immunity that are responsible for the progression of AK to invasive disease, plus other factors discussed below, have also been implicated in the progression of SCC in situ to invasive disease.

Development of BD and progression into invasive SCC

Bowen’s disease, Morbus Bowen and SCC in situ are synonymous terms for localised neoplastic degeneration limited to the epidermis. Several studies have shown the potential for these lesions to progress to invasive SCC. Most studies suggest a 3–5% risk of ‘ordinary’ BD transforming into invasive carcinoma14,15 and up to 10% for erythroplasia of Queyrat.16 In the immunosuppressed patient population, the rate of invasive disease is likely to be even higher.

A number of different aetiological factors have been described for BD including irradiation (solar, photochemotherapy, radiotherapy), carcinogens specifically arsenic, immunosuppression (therapeutic and AIDS), chronic injury, and HPV.17 As it remains impossible to identify individual lesions that may progress to invasive SCC, it is imperative to intervene as early as possible, treating the AK field of cutaneous lesions (both visible and sub-clinical) in the hope of preventing disease-associated mortality.

Treatment options for AK and BD

The European Dermatology Forum (EDF) guidelines indicate that the evidence level for using ‘destructive’ treatments (e.g. curettage, laser therapy, cryotherapy) in AK is limited to cohort and case-control studies.18,19 These guidelines also highlight that such therapeutic strategies only target single, visible lesions and not the whole AK field. As a result they are associated with recurrence rates as high as 12% in the first year post treatment.20

The rationale for non-ablative, topical therapies in the management of AK is clear. They can be easily applied to all areas of the skin, and are particularly useful in meeting the clinical need of treating lesions in cosmically-sensitive or difficult-to-treat locations. In addition, they have the potential to reduce the incidence of pain and the risk of infection and scarring. Importantly in the treatment of the AK field, these topical therapies are able to treat both visible and subclinical lesions.

The EDF AK treatment guidelines18,19 and the guidelines for treating BD (2006 update)17 provide comprehensive and concise reviews of current treatment options targeted for individual lesions. However, in the light of field cancerisation and our current, albeit limited, knowledge of tumour progression, we present a case series of five patients treated with diclofenac 3% gel (SolarazeTM) that highlights the importance of treating the field in which tumours arise, and targeting mechanisms that will halt tumour invasion.

Diclofenac 3% gel is applied locally to the skin twice daily. Normally, 0·5 g is sufficient to treat a 25 cm² lesion site. The usual duration of therapy is 60–90 days. The maximum therapeutic effect in clearing AK lesions is seen 30 days after cessation of drug therapy.21 In clinical studies, the response rate associated with diclofenac 3% gel (twice daily for 3 months) was significantly greater than with placebo (79% vs. 45%, respectively), as was complete clearance of the AK field (50% vs. 20%, respectively; P < 0·01).22 In a bilateral comparison of the efficacy and tolerability of diclofenac 3% gel and 5% 5-fluorouracil (5-FU) cream in the treatment of AKs of the face and scalp, both demonstrated substantial efficacy in the number of lesions cleared and the proportion of patients with significant lesion clearing.23 Diclofenac 3% gel induced only
mild signs of inflammation compared to 5-FU despite a longer treatment period.

Some clinicians advocate the use of a combination of destructive and non-ablative therapies as the most effective strategy for eradicating both visible and subclinical AK lesions. In the case of BD, there is good evidence to support the use of destructive therapies (excision, photodynamic therapy and curettage with electrocautery).\textsuperscript{17} Destructive therapies, however, are not always an option in every situation. Consequently, although not formally evaluated in a clinical trial setting, we propose, based on the mechanism of action and the need to treat the AK field, that diclofenac 3% gel may be a safe and effective treatment option in selected patients.

The choice of treatment for BD is guided by size and location of the tumour. Dawe et al. presented the first two cases of BD successfully treated topically with diclofenac 3% gel. The patients were treated twice daily over 80–90 days and showed both clinical and histological clearance.\textsuperscript{24} To date in our hospital, we have treated five biopsy proven BD patients using diclofenac 3% gel once daily for 8 weeks. After 6 weeks of treatment all patients developed a mild inflammation in the treated area. Dryness and itching were the most commonly reported side effects. Four weeks after the end of treatment, biopsies were repeated and revealed no clinical or histological residual disease. These results add support to Dawe’s observation that topical diclofenac 3% gel may be a useful addition to the array of therapies available for treating BD. Further studies are needed, however, to determine optimum dosage regimens and long-term follow-up.

Conclusions

Today, the mode of action of diclofenac 3% gel is much better understood. Inhibition of the COX enzymes results in a decrease in the downstream by-products of arachidonic acid (AA) metabolism. These AA metabolites have been shown to play a pivotal role in promoting epithelial tumour growth by stimulating angiogenesis, mediating the conversion of procarcinogens to carcinogens, inhibiting apoptosis and immune surveillance, and increasing invasiveness of tumour cells.\textsuperscript{11}

It has been demonstrated that treatment with diclofenac 3% gel inhibits epithelial tumour growth in AK.\textsuperscript{25} Diclofenac 3% gel induces apoptosis (not necrosis), which facilitates DNA repair mechanisms in AK.\textsuperscript{26} It has been suggested that the mode of action may be mediated by an effect on matrix metalloproteinases\textsuperscript{27} or by partial agonism of the nuclear peroxisome proliferator-activated receptor-\textgamma (PPAR-\textgamma), which plays a role in cellular differentiation and apoptosis.\textsuperscript{28} It has also been shown to inhibit tumour angiogenesis.\textsuperscript{10} This beneficial effect on tumour growth is only observed with the combination of diclofenac and hyaluronic acid (diclofenac 3% gel) and not with diclofenac alone.

The AK field involves both visible and subclinical lesions. As it is impossible to identify which of the early AK lesions will progress to SCC, the whole AK field must be treated. The significance of AK and its management is recognised in the new EDF guidelines.\textsuperscript{18} Therapeutic interventions, such as diclofenac 3% gel, are particularly suitable for treating the field cancerisation of multiple AK lesions, and for potentially eradicating BD, thereby halting the potential for tumour invasion.

At an early stage in the development of invasive SCC, the malignant potential of AKs and BD is evident, and poses a threat that primary and secondary care clinicians cannot afford to ignore. With an effective and well-tolerated, first-line topical therapy for the treatment of mild-to-moderate AK and BD lesions now available, clinicians have an unique opportunity to stop AK/BD in its tracks before it progresses to invasive full-scale SCC.

References

Genetically determined susceptibility to COX-2 inhibitors – a report of exaggerated responders to diclofenac 3% gel in the treatment of actinic keratoses

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Summary

Diclofenac 3% gel is an effective treatment for actinic keratoses (AKs) and is reported to be generally well tolerated with only mild local reactions. However, there is a subset of patients that seem to be susceptible to developing severe local reactions following application of diclofenac 3% gel. Although some of these reactions can be explained as being allergic contact dermatitis and/or phototoxic contact dermatitis, others cannot. We report a series of 10 patients who all developed severe local reactions following application of diclofenac 3% gel, despite negative diclofenac patch testing. This raises the question as to whether there is a subset of patients with skin cancer or AK lesions that are highly/more susceptible to local reactions caused by cyclo-oxygenase-2 (COX-2) inhibitors and peroxisome proliferator-activated receptor (PPAR) agonists? We speculate that underlying molecular differences exist in these patients that make the skin more susceptible to COX-2 inhibitors.

Actinic keratoses (AKs) are relatively common lesions that primarily develop in fair-skinned individuals as a result of excessive sun exposure. The importance of the malignant potential of these lesions is well known.1–3 These lesions tend to occur as multiples and it was Slaughter et al., in 1953, who first proposed the concept of field cancerisation in an attempt to explain the development of multiple primary tumours and locally recurrent cancer in oral stratified squamous epithelium.4 Recent molecular findings suggest that the clinically normal appearing tissue around AKs and squamous cell carcinomas (SCCs) is in fact a clonal expansion of a genetically altered field of pre-neoplastic cells.5 With this in mind, the diagnosis and treatment of epithelial cancers should not only focus on the tumours, but also the area of surrounding skin.

A wide spectrum of treatment modalities are available for the treatment of actinic keratosis (AK), including topical diclofenac 3% gel (Solaraze™), imiquimod 5% cream, 5-fluorouracil (5-FU), photodynamic therapy and ablative treatments, such as cryotherapy and laser therapy. Patient discomfort associated with some of these options, especially cryotherapy has driven the search for more effective and better tolerated treatments. Diclofenac 3% gel, a cyclo-oxygenase-2 (COX-2) inhibitor has been shown to be better tolerated than 5-FU and in a study comparing these two treatments, diclofenac 3% gel induced milder signs of inflammation than 5-FU, despite a more prolonged treatment period.6 Furthermore, a greater number of patients expressed significant satisfaction with diclofenac 3% gel than with 5-FU.6

Diclofenac 3% gel is generally mild and well-tolerated with a reported 51% of patients who had experienced at least one drug-related adverse event, including dry skin, rash, erythema and vesiculobullous eruptions at the site of application.7 However, several incidences of severe or strong reactions to the drug have been reported. Case reports of allergic contact dermatitis to diclofenac 3% gel date back to 1992 and since then more than 10 reports of contact dermatitis8–13 and two reports of photoallergic contact dermatitis have been documented.14,15 Until now, all of the severe local reactions reported in the literature have been attributed to allergic contact dermatitis. In our experience, however, we have found that there is a subset of patients that develop severe local reactions that cannot be explained as being contact allergic dermatitis.

Here, we present a case series of 10 patients who developed moderate-to-severe local reactions to diclofenac 3% gel. Patch testing for allergic contact reaction proved negative and all patients showed clinical clearance of their AKs. We hypothesize that underlying molecular differences exist in these patients and that lesional aberrations make the skin more susceptible to COX-2 inhibitors.

Patients and methods

Ten patients with moderate-to-severe reactions to diclofenac 3% gel were selected for patch testing between January 2005 and December 2006. The areas treated with diclofenac 3% gel varied and included the scalp, face and upper extremities.
Patients were instructed to apply the medication twice a day for up to 88 days to the documented areas affected by AK and were educated on strict sun protective measures during the course of treatment. The 10 patients selected had previously tried other treatments for their AKs without complete clearance. Actinic keratoses were graded on a scale of 1 to 3 according to Olsen et al.\textsuperscript{16} Patients were educated on the expected side effects as described in the package insert. All the patients were found to have moderate-to-severe inflammation on routine follow-up at the end of the treatment period. Moderate inflammation included patients with intense erythema and scaling, and severe inflammation was defined as intense erythema and scaling with the addition of oedema and exudative crusting. Patch tests were performed using manufacturer produced diclofenac 3% gel on the medial forearm of patients. Patch tests were read on Day 2 and Day 4. In addition, photo-patch testing was performed in two patients who reported non-compliance with sun protection methods during the treatment period.

### Observations

All ten patients had clinically evident AKs at the initiation of treatment (Fig. 1). Each patient showed moderate-to-severe erythema, scaling and crusting after initiating therapy with diclofenac 3% gel (Fig. 2). Reaction times varied from a few days after the initiation of therapy to a few weeks. On Day 2 and Day 4 patch tests were negative in all patients (Fig. 3). In addition, photopatch testing in patients 8 and 9 were negative. Table 1 summarises the patient demographics and results. All patients showed partial to complete clinical clearance of their AKs (Fig. 4).

### Discussion

Diclofenac 3% gel is a nonsteroidal anti-inflammatory drug (NSAID) formulated as 3% diclofenac sodium in 2.5% hyaluronic acid. Placebo-controlled clinical trials have demonstrated that diclofenac 3% gel is effective in the treatment of AK with minimal adverse reactions.\textsuperscript{17} In this small case series, we observed that there is a subset of patients that develop severe local reactions paralleled by clinical clearance of AKs. Interestingly, all patients demonstrated negative diclofenac patch tests. These findings support the observations made by Ortonne et al.,\textsuperscript{18} who showed that no phototoxic or photosensitisation reactions occurred with diclofenac 3% gel either alone or in combination with sunscreens. There must, therefore, be another explanation for the existence of this subset of patients who are more susceptible to local reactions. We hypothesize that variable expression of peroxisome proliferator-activated receptors (PPAR) and matrix metalloproteinases (MMP), via COX, may cause high responses to COX inhibitors. Based on these molecular differences certain subsets of patients may be more responsive to this therapy.

There are many critical steps involved in malignant tumour genesis, including cell proliferation, evasion of apoptosis, stimulation of angiogenesis, cell motility and evasion of immunosuppression. Many of these essential steps in carcinogenesis have been associated with COX-2 expression. Cyclooxygenase is the rate limiting enzyme that catalyses the formation of prostaglandins. Numerous studies indicate that COX-2 is highly expressed in a variety of human cancers, including colorectal, breast, prostate and skin cancer. In addition, the expression of the COX-2 gene has been associated with high-grade tumours of the breast and an unfavourable prognosis.\textsuperscript{19} These observations have led to studies investigating the use of COX inhibitors as a therapeutic option for cancer prevention and therapy. In 1978, Lynch et al.\textsuperscript{20} first proposed mechanisms by which aspirin and indomethacin inhibit tumour growth. Since then, NSAIDs have been shown to be effective chemoprevention agents for colon cancer,\textsuperscript{21} breast cancer\textsuperscript{12} and SCC/AK.\textsuperscript{23,24}
The mechanism of action of NSAIDs in treating cancer, including the effects of diclofenac 3% gel in AK, is not yet fully understood. Diclofenac, however, has been shown to have at least two mechanisms of actions; the inhibition of COX enzymes and matrix metalloproteinases (MMPs), and COX-independent actions via PPAR. The MMPs constitute a family of highly homologous extracellular zinc- and calcium-dependent endopeptidases with enzymatic activity against almost all protein components of the extracellular matrix.

In order for cancer cells to metastasise, they must digest and dissolve the extracellular matrix and the basement membrane, which requires the presence of active MMPs. Recent data suggest a correlation between COX-2 expression and cell invasiveness, thereby leading researchers to investigate whether MMPs mediate tumour invasion in a COX-dependent fashion. Sivula et al. found increased COX-2 expression in breast cancer specimens that exhibited elevated MMP-2 expression and an overall decrease in disease-specific survival. Also in breast cancer, Larkin et al. reported that inhibition of COX-2 decreased cell motility, invasion and MMP expression. COX-2 is not expressed in a normal membrane, but its expression is induced by stimuli, such as inflammation and inflammatory cytokines. Cyclo-oxygenase-2 over expression has already been demonstrated in skin tumours, including SCCs and AKs. Increased inflammation, as seen in our patients, may potentially upregulate greater COX-2 expression and thereby, make COX inhibition more effective by down regulating the cytokines and MMPs, which allow tumour genesis and invasion. Thus variable expression of COX and, thereby, COX inhibition in skin, could account for our observations.

There is evidence that NSAIDs may act as chemoprotective agents through targets other than COX. Peroxisome proliferator-activated receptor- is one such target that has been shown to have a role in inhibiting cancer cell growth. In addition to controlling cell differentiation, proliferation and apoptosis, PPAR ligands also have anti-inflammatory properties and inhibit angiogenesis. A recent retrospective immunohistochemical analysis of normal skin, AK, and SCC showed that PPAR- was significantly less likely to occur in SCC and AK than in normal skin, and PPAR- appeared to be upregulated in premalignant skin. In addition, COX-2 immunopositivity was significantly associated with PPAR- and PPAR- immunoreactivity. These findings support our hypothesis that variable expression of receptors may mitigate varied responses to COX inhibitors.

It would appear that there is a molecular basis for the existence of the subset of patients reported here. Our results are limited, however, as no molecular studies were performed to support our hypothesis. However, based on reports from other carcinomas and following our clinical observations, further investigations should be carried out. We feel that in the future, patients with severe local reactions to diclofenac 3% gel should undergo patch and photopatch testing as this may provide the key to determining which lesions will respond to topical COX inhibitors. Just as the discovery of oestrogen receptor positivity has revolutionised the treatment of breast cancer, we feel further investigations into the genetic susceptibility of skin tumours will change the way we treat AKs and SCCs in the future, and allow treatment approaches to be tailored to appropriate patients.

In the setting of field cancerisation, the potential to not only treat existing cancers, but to also halt the progression to invasive disease, offers new treatment avenues based on old therapeutic options. We believe our clinical observation raises the possibility of there being differences at the molecular level.
and that further investigations into the molecular basis of AK and cutaneous SCC will help to elucidate the mechanisms of tumour invasion and offer improved screening and treatment options for patients.

### References


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Table 1: Patient demographics and results

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Sex/Age (years)</th>
<th>AK location</th>
<th>Extent of AK at initiation of therapy</th>
<th>Therapy duration (days)</th>
<th>Grading of inflammatory response</th>
<th>Patch test</th>
<th>Photo patch test</th>
<th>Clearance of AKs</th>
<th>Patch test</th>
<th>Extent of AK at initiation of therapy</th>
<th>Therapy duration (days)</th>
<th>Grading of inflammatory response</th>
<th>Patch test</th>
<th>Photo patch test</th>
<th>Clearance of AKs</th>
<th>Patch test</th>
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<tbody>
<tr>
<td>1</td>
<td>M/60</td>
<td>Forehead, nose, temples, cheeks</td>
<td>Moderate</td>
<td>2</td>
<td>Moderate</td>
<td>Negative</td>
<td>ND</td>
<td>CR</td>
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<tr>
<td>2</td>
<td>F/46</td>
<td>Nose, cheeks, temples</td>
<td>Mild</td>
<td>1 to 2</td>
<td>Moderate</td>
<td>Negative</td>
<td>ND</td>
<td>CR</td>
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<tr>
<td>3</td>
<td>M/72</td>
<td>Complete face: forehead, nose, temples, cheeks</td>
<td>Severe</td>
<td>14</td>
<td>Moderate</td>
<td>Negative</td>
<td>ND</td>
<td>CR</td>
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<td>Negative</td>
<td>ND</td>
<td>CR</td>
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<td>Moderate</td>
<td>Negative</td>
<td>ND</td>
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<td>6</td>
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<td>1 to 2</td>
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<td>ND</td>
<td>CR</td>
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<td>ND</td>
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<tr>
<td>9</td>
<td>M/69</td>
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<td>2 to 3</td>
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<td>ND</td>
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AK, actinic keratoses; ND, not done; CR, complete response; PR, partial response.

**Table 1** Post-treatment photograph showing clearance of AKs and severe inflammation.
18 Ortonne JP, Queille-Roussel C, Duteil L. 3% diclofenac in 2.5% hyaluronic acid (Solaraze) does not induce photosensitivity or phototoxicity alone or in combination with sunscreens. Eur J Dermatol 2006; 16:385–90.