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Cell Apoptosis as Assessed by M30 Expression in Keratoacanthoma and Squamous Cell Carcinoma

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A B S T R A C T

Involution displayed by keratoacanthoma (KA) represents an important difference between KA and squamous cell carcinoma (SCC). It has been suggested that apoptosis plays a part in process of involution of KA. Altogether 150 specimens were included in this study, 30 cases of each; normal skin (NS), proliferative (pKA) and regressing keratoacanthoma (rKA), well differentiated (wdSCC) and poorly differentiated (pdSCC) squamous cell carcinoma. All samples were examined immunohistochemicaly for expression of M30 protein. A significantly lower number of M30 positive cells has been detected in NS as compared to skin tumors examined ($p<0.001$), except for rKA ($p=0.057$). The highest percentage of M30 positive cells was detected in pdSCC ($p<0.001$) as compared with all other examined groups. Keratinocytes of normal and changed epidermis expressing higher levels of M30 protein were predominately found in sun-exposed areas ($\chi^2=14.93$; $p=0.060$). There was an increasing trend of M30 protein expression with increasing age of the patient in NS and skin tumors examined. Majority of skin tumors with higher percentage of M30 positive cells tended to display higher Ki-67 expression. M30 expression was highly correlated with bak ($r=0.811$; $p=0.048$) and granzyme B expression in rKA ($r=0.733$; $p=0.015$). Cell apoptosis as assessed by M30 expression is, generally, increased in examined skin tumors and related to cell proliferation. Cell apoptosis mediated by bak and granzyme B expression could contribute to KA regression.

Key words: *apoptosis, keratoacanthoma, M30, squamous cell carcinoma*

Introduction

The distinction of keratoacanthoma (KA) from squamous cell carcinoma (SCC) on histological grounds has been a matter of convention. KA is an unusual cutaneous neoplasm characterized by self-involution[1–3].

Molecular events regulating cell survival, apoptosis, growth arrest and cell differentiation have been shown to contribute to the overall kinetics and progression or regression of benign and malignant cell growth[4–6]. Apoptosis, or programmed cell death, is the death of individual cells by a genetically controlled mechanism[7]. Apoptosis occurs in many physiological as well as in a variety of pathologic conditions and has been reported to significantly influence the growth rate of tumors[5,8–10]. Cell lines with high apoptotic rate *in vitro* tend to form slower growing tumors than those with low apoptotic rates[11]. Previously, we have suggested a possible contributory role of altered Bcl-2 proteins expression[12] and immunologic mechanisms in the process of KA regression[13]. Previous researches implicated a possible role of immunological mechanisms in a phenomenon of spontaneous tumor regression[8,11].

To investigate further and to assess the possible differences in apoptotic activity between KAs and SCCs as well as the significance of apoptosis in mediating KA regression we have examined immunohistochemicaly the expression of the specific caspase cleavage site within cytokeratin 18, not detectable in native cytokeratin 18 of normal cells, using monoclonal antibody M30 CytoDeath[14] in regressing (rKA) and proliferating (pKA) KAs,

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well differentiated (wdSCCs) and poorly differentiated SCCs (pdSCC). In order to correlate obtained results with the results of our previous researchers and to determine the role of Bcl-2 family proteins and granzyme B mediated cytotoxicity in KA regression, immunohistochemical analyses were performed using same normal skin and skin tumor samples that have been used in during our previous researches[12,13].

Materials and Methods

Patients and skin specimens

150 of skin specimens were obtained from patients at Department of Dermatovenerology, University Hospital Rijeka and Department of Pathology, Faculty of Medicine, University of Rijeka, Croatia between 2000 and 2005. Specimens included 30 cases of each; normal skin (NS), proliferative keratoacanthoma (pKA), regressing keratoacanthoma (rKA), well differentiated (wdSCC) and poorly differentiated squamous cell carcinoma (pdSCC). In this study we classified SCCs into two categories, according to Broders' grading; well (Broders' grade I) and poorly (Broders' grade III) differentiated type. NS samples were obtained from normal skin surrounding fibromas or hemangiomas surgically resected for cosmetic reasons.

Tissues were obtained from 82 (54.67%) men and 68 (45.33%) women. There was no difference in sex of tested patients (χ^2=0.43; p=0.979). Average age of the patients was 69.10 years (range 48–89 years), 67.87 years (range 49–86 years), 75.50 years (range 60–91 years), 76.57 years (range 60–92 years), 65.86 years (range 39–87 years) for pKA, rKA, wdSCC, pdSCC and healthy controls, respectively. The patients with NS were significantly younger as compared to patients with wdSCC and pdSCC (p<0.001). All specimens were fixed in 10% buffered formaldehyde and embedded in paraffin. 4μm-thick sections were stained with hematoxylin-eosin and two pathologists examined each slide independently.

Histology

Both pKA and rKA were histologicaly determined according to previously listed criteria[12,13]. In brief, both pKA and rKA were characterized by the presence of an exoendophytoc squamous proliferation with a central, keratin-filled crater with the overlying epidermis extended around the crater forming »lips«. The pKA was predominantly composed of the well differentiated proliferative epithelium enveloping a small, keratin filled crater surrounded by moderate to intense lymphohistiocytic inflammation at the base. With progressive regression of the lesion (rKA), the keratin filled crater was diminished and the proliferating epithelium tended to flatten out, with diminished inflammation and fibrosis underlying the base.

Immunohistochemical staining

The primary antibody solution was a 1:50 dilution of M30 (Roche Diagnostics GmbH, Mannheim, Germany). A colonic adenocarcinoma specimen served as positive control samples. Additional sections were run parallel with omission of the primary antibodies and served as negative control.

M30 immunostaining was performed as follows. Paraffin-embedded tissue sections were deparaffinized in xylene and rehydrated by washing in absolute and diluted ethyl alcohol and distilled water. Staining was carried out after sections were treated for antigen retrieval according to manufacturer's instructions. This was followed by the standard ABC (avidin-biotin complex) procedure for 2 hours and 10 minutes in DAKO Techmate Immunostainer (Techmate Horizon, serial No. 30097, LJL Biosystems Inc., USA).

The sections were examined at high power, with at least 1000 cells being counted in each case. Cells exhibiting immunoreactivity for M30 were expressed as a percentage of the total number of epithelial cells counted in each section. Counting fields were randomly selected to minimize possible bias.

Statistics

The values of M30 protein expression were expressed as median, quartiles and percentiles because of the distribution of the results previously tested by Kolmogorov-Smirnov test. The differences in age and sex were analyzed by one-way ANOVA and Chi-quadrat test, respectively. The differences in M30 expression between the groups were analyzed using the Kruskal-Wallis test and as a post-hoc test Mann-Whithey U test. Association of parameters was tested using Pearson's correlation coefficient, with linear regression whenever a significant correlation was found. P values of less than 0.05 were considered statistically significant.

TABLE 1
EXPRESSION OF M30 PROTEIN IN KERATOACANTHOMA AND SQUAMOUS CELL CARCINOMA

Diagnosis	Median (%)	IQR (%)	p*
Proliferative keratoacanthoma	0.85	(0.5–1.1)	<0.001
Regressing keratoacanthoma	0.45	(0.2–0.7)	
Well differentiated squamous cell carcinoma	1.35	(1.1–1.6)	
Poorly differentiated squamous cell carcinoma	2.80	(1.5–3.4)	
Normal skin	0.25	(0.0–0.6)	

IQR – interquartille ranges; χ^2 test *p<0.05

Results

Immunohistochemical staining for M30 protein was detected in 29 (96.67 %) cases of pKA, 26 (86.67 %) cases of rKA, 30 (100.00%) cases of wdSCC, 30 (100.00%) cases of pdSCC and 17 (56.67 %) cases of healthy controls. Median values and quartiles (IQR) detected are shown in Table 1.

Apoptotic M30 positive cells were extremely rare in the normal epidermis. Only a few apoptotic cells were detected in the upper portion of the epidermis (not shown). In KAs M30 positive cells could be detected mainly in the upper epidermis overlying central, keratin-filled crater as well as in the basal layer in the contact with surrounding inflammatory infiltrate (Figure 1a, 1b). M30 positive cells showed no predominant distribution in pdSCC lesions while apoptotic cells were more prominent near keratinizing foci and at the front of the tumor invasion in wdSCC (Figure 2a, 2b), as shown previously[9]. We have detected a significantly lower number of M30 positive cells in NS as compared to skin tumors examined ($p< 0.001$), except for rKA ($p=0.057$). Contrary, the highest percentage of M30 positive cells was detected in pdSCC ($p<0.001$) as compared with all other examined groups.

There was a significant difference in M30 expression depending on lesion localization ($\chi^2=14.93$; $p=0.060$). Overall, keratinocytes of normal and changed epidermis expressing higher levels of M30 protein were predominately found in sun-exposed areas (head and neck) (Table 2). There was an increasing trend of M30 protein expression with increasing age of the patient in NS ($r=0.773$; $p<0.001$), pKA ($r=0.559$; $p=0.001$), wdSCC ($r=0.585$; $p=0.0007$) and pdSCC ($r=0.732$; $p<0.0001$) and rKA ($r=0.279$; $p=0.135$). Overall, keratinocytes of normal and altered epidermis expressing higher levels of M30 protein were predominately found in older individuals.

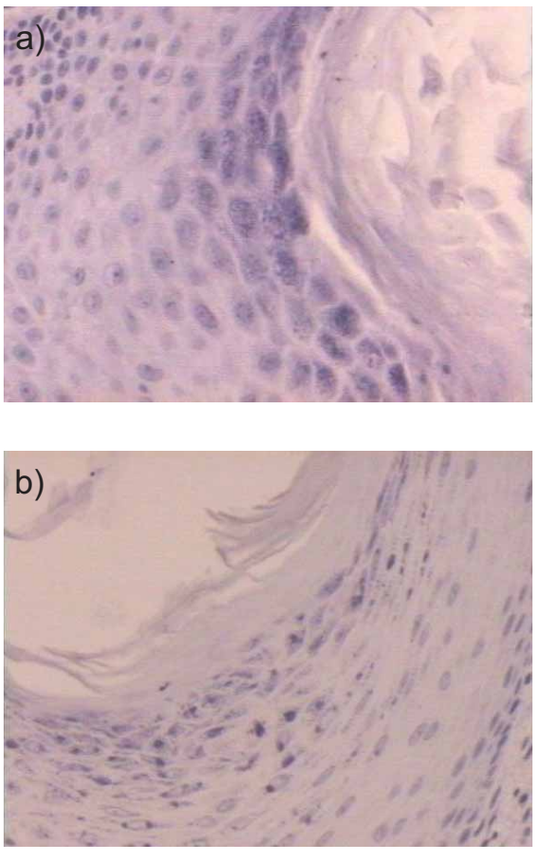

Fig. 1. Immunohistochemical staining for M30 protein in proliferative and regressing keratoacanthoma. a) Immunohistochemical staining of M30 protein in proliferative keratoacanthoma. Bak protein was expressed strongly in proliferative keratoacanthoma. Apoptotic cells could be seen mainly in the epidermis overlying central, keratin-filled crater (HEx400). b) Immunohistochemical staining of M30 protein in regressing keratoacanthoma. A few M30 positive cells could be detected mainly in the epidermis overlying central, keratin-filled crater as well as in the basal layer in the contact with surrounding inflammatory infiltrate (HEx200).

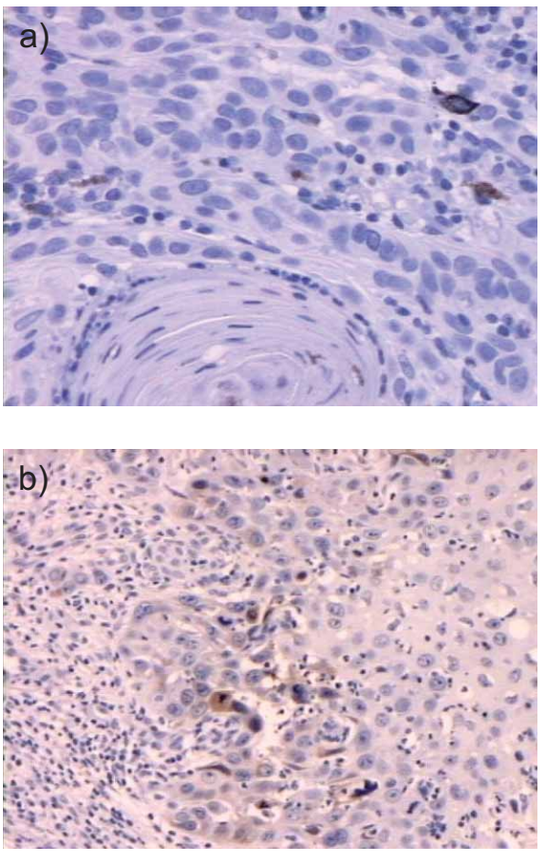

Figure 2. Immunohistochemical staining for M30 protein in well differentiated and poorly differentiated squamous cell carcinoma. a) Immunohistochemical staining of M30 protein in well differentiated squamous cell carcinoma. M30 positive cells could be seen in diffuse pattern through out the lesion but were more prominent near keratinizing foci and at the front of the tumor invasion (HEx200). b) Strong immunohistochemical staining of M30 protein in poorly differentiated squamous cell carcinoma with numerous positive cells distributed in a diffuse pattern (HEx100).

TABLE 2
EXPRESSION OF M30 PROTEIN IN KERATOACANTHOMA AND SQUAMOUS CELL CARCINOMA ACCORDING TO LESION LOCALIZATION

Diagnosis	Head/neck		Extremities		Trunk		p*
	N	Median (5–95th)%	N	Median (5–95t^{h})%	N	Median (5–95th)%	
Proliferative keratoacanthoma	21	0.99 (0.79–1.18)	7	0.46 (0.19–0.72)	2	0.55 (0.00–1.18)	0.009
Regressing keratoacanthoma	21	0.50 (0.36–0.63)	5	0.32 (–0.14–0.78)	4	0.35 (–0.03–0.73)	0.414
Well differentiated squamous cell carcinoma	20	1.58 (1.34–1.82)	8	1.11 (0.87–1.35)	2	1.10 (–0.17–2.37)	0.021
Poorly differentiated squamous cell carcinoma	21	2.72 (2.21–3.23)	8	2.32 (1.44–3.21)	1	1.50	0.398
Normal skin	13	0.56 (0.38–0.75)	8	0.18 (–0.05–0.40)	9	0.06 (–0.03–0.14)	0.0008

χ^2 test *p<0.05

In order to determine a possible role of different pro-apoptotic and anti-apoptotic proteins and granzyme--mediated cytotoxicity in apoptosis of tumor cell and KA regression, we have correlated obtained results with the results of our previous studies obtained by immunohistochemical staining of the same skin tumors and normal skin samples[12,13]. Majority of skin tumors with higher percentage of M30 positive cells tended to display higher Ki-67 expression with coefficient of correlation as follows: r=0.919; p=0.002 for pKA; r=0.725; p<0.001 for wdSCC; r=0.819; p=0.003 for pdSCC; except in rKA (r=0.881; p=0.675) and NS samples (r=0.763; p=0.239). M30 expression was highly correlated with bak expression in rKA (r=0.811; p=0.048) and weakly but positively correlated in NS (r=0.426; p<0.0001). Also, M30 expression has been found to be highly correlated with granzyme B expression in rKA (r=0.733; p=0.015). We have found no other significant associations between M30 expression and p53, bcl-2, bak, CD8, CD4, CD3, and granzyme B expression in skin tumors examined and NS samples.

Discussion

Apoptosis has been suggested to play a part in KA involution process[5,9,12,13], although exact trigger and mechanisms involved remain unclear. The majority of the lesions that clinically and/or histologicaly fit diagnosis of KA behave in a predictably benign manner, but occasional aggressive behavior, including metastases, has been described[3,15,16]. Thus, it is still not uniformly accepted weather KA is a variant of SCC or a unique lesion[1–3].

We have demonstrated cell loss by apoptosis in a spectrum of skin squamous neoplasia, including pKA, rKA, wdSCC and pdSCC in order to determine the possible differences and the role of cell apoptosis in KA regression. The number of apoptotic cells, as assessed by M30 expression, increased with increasing malignant potential, the highest value being noted in pdSCC, and the lowest values in rKA as well as in NS. Previously, Makino et al. and Einspahr et al. reported increased number of apoptotic cells in poorly differentiated skin SCCs in correlation with cell proliferation[9,17]. Also, Isacson at al. found that both cell proliferation and apoptosis increased with lesion grade in cervical neoplasia, regardless of the human papilloma virus type[18].

It has been suggested that apoptosis might reflect not only cell loss but also cell proliferation activity in the squamous cell neoplasia of the skin and cervix uteri. We have shown previously that proliferation, as assessed by Ki-67 expression, was significantly higher in pdSCC as compared with pKA, but intensity of cell proliferation was similar in wdSCC and pKA[12]. In order to determine the association between cell proliferation and cell apoptosis we have correlated obtained results with previously determined cell proliferation. Generally, the number of M30 positive cells increased with increasing cell proliferation in SCCs and pKA. These results suggest that increased proliferation stimulates apoptosis in the majority of skin cancers as suggested previously[17]. Low M30 expression as well as the lack of correlation between M30 and Ki-67 expression found in NS and rKA, could be due to low cell proliferation intensity detected in these tissues.

Intensive apoptosis could contribute to clinically slow growth and development of SCC lesions counteracting intensive cell proliferation[2,3,12]. On the other hand, relatively low apoptosis level, in association with previously determined high proliferation rate[12], could contribute to the initial intensive development seen in pKA. In the light of obtained results we can also suggest that low level of cell apoptosis found in rKA could not solely, but only in association with decreased cell proliferation, contribute to KA regression.

Keratinocytes of normal and changed epidermis expressing higher levels of M30 protein were predominately found in sun-exposed areas and in the older individuals as shown previously for p53, bak and Ki-67 protein expression[4,12]. Increased M30 expression could be due to previously suggested increased spontaneous apoptosis during UV-induced skin carcinogenesis[17] or a response to UV-irradiation induced keratinocyte proliferation[19].

Apoptosis has been reported to significantly influence growth rate of tumors. Bcl-2 and its homologous proteins have emerged as one of the most important regulators of programmed cell death[20–23]. The pro-survival Bcl-2 family proteins, such as bcl-2/bcl-x block apoptosis, whereas Bax-like proteins bax and bak are considered to be essential for apoptosis execution[20,21,25].

Our results suggest that cell apoptosis in rKA and NS is, at least partly, bak dependent, contrary to SCC and pKA where apoptosis is, probably, due to intensive cell proliferation. We have shown that M30 expression is considerably increased in SCCs, especially in pdSCC, Generally, SCCs stains only weakly for pro-apoptotic bak and bax proteins, contrary to strong expression found in KAs[6,12,26,27]. Decreased bak expression has been linked to decreased number of apoptotic cells and continuous expression of proliferation markers in skin cancer, and its altered expression has been associated with HPV E6 protein[6,27]. So, we suggest the possible role of other apoptotic mechanisms operating in these tumors, such as Fas/FasL mechanisms.

We have found that M30 expression is positively correlated with bak expression in rKA and NS suggesting a potential role of increased bak protein expression in KA regression as well as its role in regulation of cell apoptosis and proliferation in healthy epidermis. On the other hand, we cannot forget that previous researches have shown high levels of bak expression to correlate with good and poor prognosis or had no prognostic significance[26–29].

Protein p53 is a well-described tumor suppressor that has a central role in the initiation of apoptosis and in cell cycle control[25,30]. Protein p53 expression, as measured by immunohistochemistry, has been reported in numerous studies with extremely variable results[4,9,31]. Also, it has been shown that aberrant p53 expression does not correlate with the number of apoptotic cells observed in SCCs[6,29,31] confirming the presence of p53-dependent and p53-independent mechanisms[25,32]. Similarly, we have found no correlation between number of apoptotic cells as expressed by M30, and p53 protein expression in KAs, SCCs and healthy epidermis. Some studies detected negative correlation between p53 protein expression and apoptosis[31].

Previous studies detected a modest expression of bcl-2 protein in all squamoproliferative lesions[12,17,33]. Although, decreased bcl-2 expression has been suggested to play a possible role in KA regression, we have detected no correlation between M30 and bcl-2 expression in KAs, SCCs or NS. These findings are in agreement with findings in oral[29] or laringeal squamous cell carcinoma[33].

A significant correlation between the number of apoptotic cells and the number of T cells expressing granzyme B found in rKA, speaks in favor of previously suggested significance of granzyme B mediated cytotoxicity in KA regression[13]. In other examined skin tumors and healthy skin there was a tendency of increased M30 expression with increasing granzyme B expression but the correlation did not reach statistical significance suggesting a possible contributory role but also involvement of other pro-apoptotic or anti-apoptotic factors in cell proliferation control. On the other hand, we have detected no such association between M30 expression and the number of CD3+, CD4+ and CD8+ cells.

In conclusion, cell apoptosis as assessed by M30 expression is, generally, increased in examined skin tumors as compared with NS and related to cell proliferation. Increased cell apoptosis detected in pKA could contribute to tumor regression in conjunction with decreased cell proliferation during tumor maturation. We have shown that increased bak and granzyme B expression have a role in cell apoptosis in rKA. Obtained results give us a better insight into the pathogeneses mechanisms involved in skin cancer development, progression and in some cases regression. In biological drugs and specific antibodies era, each of proposed apoptotic molecules is a potential new therapeutic target that will enable more specific treatment.

Many studies have been undertaken in order to develop a simple method for differentiation between KA and SCC in everyday practice, since the distinction between these two skin tumors, on histological grounds only, could sometimes be difficult. We have shown that cell apoptosis as assessed by M30 expression is related to malignant potential in examined skin tumors, as well as with cell proliferation, thus this method could aid in differentiation between these two closely related entities.

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EKSPRESIJA M30 PROTEINA KOD KERATOKANTOMA I SPINOCELULARNOG KARCINOMA

S A Ž E T A K

Regresija keratoakantoma (KA) temeljna je razlika između KA I spinocelularnog Karcinoma kože (SCC). Sugerirano je da programirana smrt stanice (apoptoza) ima ulogu u procesu involucije KA iako točni pokretači i mehanizmi djelovanja nisu u potpunosti razjašnjeni. Analizirano je 150 uzoraka, po 30 uzoraka; zdrave (NS), keratoakantoma u stadiju proliferacije (pKA) i regresije (rKA), dobro diferenciranog (wdSCC) i slabo diferenciranog (pdSCC) spinocelularnog karcinoma. Svi su uzorci analizirani imunohistokemijski kako bi se utvrdila ekspresija M30 proteina. Utvrđen je značajno niži broj M30 pozitivnih stanica u uzorcima zdrave kože u usporedbi s ispitivanim tumorima kože ($p<0,001$), osim u rKA ($p=0,057$). Najviši postotak M30 pozitivnih stanica nađen je u pdSCC ($p<0,001$) u odnosu prema svim ispitivanim skupinama uzoraka. Keratinociti zdravog i izmijenjenog epidermisa s utvrđenim višim vrijednostima ekspresije M30 proteina pretežno su bili sa suncu-izloženih dijelova tijela ($\chi^2=14,93$; $p=0,060$). Utvrđen je pozitivan trend ekspresije M30 proteina s porastom dobi ispitanika u svim ispitivanim skupinama. U tumora s intenzivnijom proliferacijom stanica utvrđen je viši postotak M30 pozitivnih stanica. Ekspresija M30 proteina u korelaciji je s ekspresijom bak proteina ($r=0,811$; $p=0,048$) i granzima B u rKA ($r=0,733$; $p=0,015$). Apoptoza stanica utvrđena ekspresijom M30 proteina je povišena i povezana s proliferacijom stanica u ispitivanim tumorima kože. Apoptoza posredovana bak i granzime B ekspresijom može doprinijeti regresiji KA.