

Expression of the Novel Inhibitor of Apoptosis Survivin in Normal and Neoplastic Skin

Caterina Chiodino, Anna Maria Cesinaro,* Daniela Ottani, Fabrizio Fantini, Alberto Giannetti, Gian Paolo Trentini,* and Carlo Pincelli

Department of Neuropsychosensorial Pathology, Section of Dermatology and *Department of Morphological Sciences and Legal Medicine, Section of Anatomic Pathology, University of Modena and Reggio Emilia, Italy

Apoptosis plays a fundamental part in epidermal homeostasis, and apoptotic cells have been detected in normal and diseased skin. Little is known, however, on the inhibitory mechanisms of apoptosis at the skin level. In addition to bcl-2, a novel inhibitor of apoptosis designated *survivin* and structurally analogous to IAP apoptosis inhibitors has been recently identified. The expression of survivin in normal and pathologic skin was investigated. Immunohistochemical studies revealed that survivin is expressed in basal keratinocytes, but not in suprabasal epidermal layers, with a pattern similar to bcl-2. In western blots, the anti-survivin antibody recognized a single band of 16.5 kDa in protein extracts from normal human keratinocytes in culture, in agreement with the predicted size of

survivin. In addition, survivin immunoreactivity was detected in benign and malignant melanocytic lesions, with strong expression in invasive lesions of melanomas. Whereas survivin staining was undetectable in benign epithelial tumors, such as seborrheic keratoses, it was observed in all epidermal layers in Bowen's disease. Interestingly, at variance with bcl-2, survivin was markedly expressed in squamous cell carcinoma, but virtually lacking in basal cell carcinoma, suggesting that these two apoptosis inhibitors may act through different anti-apoptotic pathways. Deregulation of survivin may influence both epidermal homeostasis and the development of melanoma and nonmelanoma skin cancer. *Key words: epidermis/IAP proteins/skin cancer. J Invest Dermatol 113:415-418, 1999*

Apoptosis, or programmed cell death, is a physiologic process of gene-directed cell suicide (Raff, 1992). Derangements of apoptosis may trigger several disease states, such as viral infection, neurodegenerative disorders and cancer (Thompson, 1995). In the skin, apoptosis plays an important part in morphogenesis (LeBrun *et al*, 1993; Polakowska *et al*, 1994) and homeostasis (Budtz, 1994; Polakowska and Haake, 1994). It has been shown recently that keratinocytes possess reversible physiologic defenses against spontaneous and ultraviolet light-induced apoptosis (Norris *et al*, 1997; Pincelli *et al*, 1997). Similarly, human melanocytes are protected from ultraviolet-induced apoptosis by neurotrophic proteins (Zhai *et al*, 1996) secreted in a paracrine fashion by other skin cells (Yaar *et al*, 1994). On the other hand, loss of normal apoptosis may be involved in the development of skin cancer. Indeed, in ultraviolet-induced skin cancer, inactivation of the tumor suppressor gene p53 reduces the appearance of "sunburn cells", apoptotic keratinocytes that might have incurred mutations and thus are to be eliminated (Ziegler *et al*, 1994). Bcl-2 is a major

anti-apoptotic protein (Kroemer, 1997) that is expressed in normal keratinocytes and melanocytes (Rodriguez-Villanueva *et al*, 1995). Aberrant expression of bcl-2 has been involved in tumor development (Fanidi *et al*, 1992), and changes in bcl-2 levels have been observed in melanoma (Kanter-Lewenshon *et al*, 1997) and nonmelanoma (Cerroni and Kerl, 1994; Morales-Ducret *et al*, 1995) skin cancers.

Recently, a family of anti-apoptotic cellular genes related to the baculovirus *iap* gene has been described (Duckett *et al*, 1996; Uren *et al*, 1996). The *iap* genes display two distinct structural features, a carboxyl-terminal RING finger and an amino-terminal repeat termed baculovirus IAP repeat (BIR) (Lovering *et al*, 1993; Clem and Miller, 1994). IAP proteins inhibit apoptosis in different contexts (Liston *et al*, 1996), although their mechanism of action is not completely understood. Recent studies show that human X-chromosome-linked IAP directly inhibit two members of the caspase family of cell death proteases (Deveraux *et al*, 1997). *Survivin* is a novel gene encoding a structurally unique IAP apoptosis inhibitor, containing a single BIR domain, but lacking the RING finger. Survivin inhibits apoptosis induced by interleukin-3 withdrawal in B cell precursors (Ambrosini *et al*, 1997). Moreover, it has been shown recently that survivin is expressed in HeLa cells in a cell-cycle regulated manner and associates with microtubules during mitosis. Disruption of survivin-microtubule interaction results in loss of survivin's anti-apoptotic function (Li *et al*, 1998). Survivin has been detected in several human cancers, such as lung, breast, colon, and pancreas adenocarcinoma and in non-Hodgkin lymphoma, whereas it has not been found in most normal adult tissues (Ambrosini *et al*, 1997, 1998). Because little is known on

Manuscript received January 28, 1999; revised May 27, 1999; accepted for publication May 28, 1999.

Reprint requests to: Dr. Carlo Pincelli, Associate Professor, Department of Neuropsychosensorial Pathology, Section of Dermatology, University of Modena and Reggio Emilia, Via del Pozzo, 71, 41100 Modena, Italy. E-mail: carlo@unimo.it

Abbreviations: IAP, inhibitor of apoptosis protein; S-IR, survivin immunoreactivity.

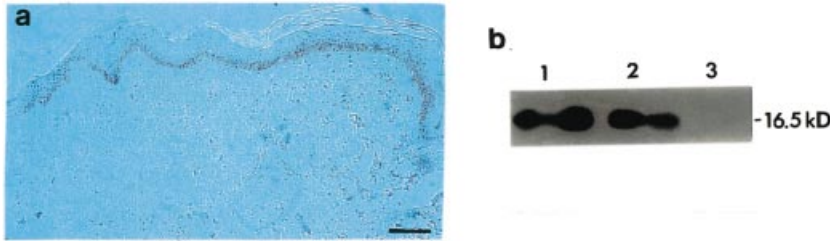


Fig 1. Expression of survivin in normal adult skin. Tissue sample was formalin-fixed, paraffin embedded, and stained with anti-survivin antibody. Scale bar: 100 μ m (a). Protein extracts from cultured normal human keratinocytes were separated on 12% polyacrylamide gels and transferred onto nitrocellulose membrane. Membranes were immunoblotted with anti-survivin antibody (lane 2) or nonimmune rabbit IgG (lane 3). Jurkat cell line was used as a positive control (lane 1) (b).

the inhibitory mechanisms of apoptosis and in particular on IAP proteins at the skin level, we investigated the expression of survivin in normal skin. In addition, we studied the expression of this protein in benign and malignant skin tumors, to evaluate the possible role of survivin in skin cancer.

MATERIALS AND METHODS

Tissue procurement A total of 56 cases of human skin retrieved from the archives of the Anatomic Pathology was used in this study. The distribution was as follows: two normal adult skin and one fetal skin, three solar keratoses, two seborrheic keratoses, one keratoacanthoma, eight basal cell carcinomas (BCC, seven nodulocystic and one superficial), four Bowen's diseases, seven squamous cell carcinomas (SCC), two junctional nevi, two dermal nevi, one compound nevus, three Spitz nevi, six *in situ* melanomas, five infiltrating melanomas (four in vertical phase, one in radial growth pattern), two cutaneous metastases of melanoma, one Merkel cell tumor, two low-grade B cell lymphomas, two T cell lymphoma, one pseudolymphoma, and one Jessner-Kanof infiltrate.

Immunohistochemistry Five micron sections cut from formalin-fixed, paraffin-embedded specimens were deparaffinized in xylene and rehydrated in graded alcohol. Quenching of the endogenous peroxidase activity was obtained by treatment with 6% H_2O_2 in methanol. The slides were first boiled in pH 6 citrate buffer for 15 min in a standard pressure cooker, then incubated with undiluted supernatant of anti-survivin monoclonal antibody (kindly supplied by Professor Dario Altieri, Department of Pathology, Yale University) at 4°C overnight. After three washes of 5 min in phosphate-buffered saline (PBS), slides were incubated with biotin-conjugated anti-mouse antibody (Linker reagent, Bioponica, Italy) for 30 min at room temperature, followed by incubation with conjugated streptavidin-horseradish peroxidase for 30 min (Tracer reagent, Bioponica, Italy). After three washes of 5 min in PBS, the slides were stained with 3',3'-diaminobenzidine for 10 min, then counterstained for 5 min with hematoxylin. In control experiments, endogenous biotin was blocked by applying avidin for 30 min, followed by biotin for 30 min (Endogenous Biotin Blocking Kit, Ventana, CA) prior to the application of the biotinylated detection reagent. Negative controls, obtained by substituting supernatant with normal serum, and positive controls (colonic adenocarcinoma) were also included in the study. A further control was performed by preabsorption of the antiserum with recombinant survivin (1.7 mg per ml).

Western blot analysis Normal human keratinocytes and Jurkat cells were washed with PBS and lysed on ice in RIPA buffer pH 8.5 (50 mM Tris-HCl, 150 mM NaCl, 1% deoxycolate, 1% TritonX-100, 0.1% SDS, 0.2% sodium azide) containing phenylmethylsulfonyl fluoride (4 mM, Sigma, St Louis, MO), aprotinin (0.2 trypsin inhibitory unit per ml, Sigma), and leupeptin (10 mg per ml, Sigma). After boiling in Laemmli sample buffer, 50 mg of total protein were analyzed under reducing conditions on 12% polyacrylamide gels and transferred on to nitrocellulose membrane. To verify equal loading of total protein in all lanes, the membrane was stained with red ponceau. The blot was blocked for 2 h in blocking buffer (PBS buffer, pH 7.4 with 0.2% Tween 20 and 5% nonfat milk) and incubated with anti-survivin or rabbit IgG antibody overnight at 4°C. Then membranes were washed in PBS/Tween 20, incubated with peroxidase-conjugated goat anti-rabbit antibody (1:800) for 45 min at room temperature, washed and developed using the ECL chemiluminescent detection system (Amersham, Milano, Italy).

RESULTS

Expression of survivin in normal adult skin Survivin immunoreactivity (S-IR) was observed as a cytoplasmic staining in the epidermal basal layer of normal human skin, the intensity

Table I. Survivin expression in pathologic skin

	No. (positive/total)	Staining intensity
Pigmented lesions		
melanocytic nevi	4/5	+ - / +
Spitz nevi	3/3	+ / + + (focal)
<i>in situ</i> MM	6/6	+ / + + (focal)
invasive MM	5/5	+ / + +
metastatic MM	2/2	+ / + +
Epithelial lesions		
solar keratoses	0/3	-
seborrheic keratoses	0/2	-
Bowen's disease	3/4	+ (all layers)
keratoacanthoma	1/1	+ - (only basal cells)
SCC	6/7	+ / + + (focal)
BCC	1/8	+ -
Others		
B cell lymphomas	1/2	- / + -
T cell lymphomas	0/2	-
pseudolymphomas	1/1	- / + -
Merkel cell carcinoma	1/1	+ - / +

- , no S-IR; + - , weak S-IR; + , moderate S-IR; + + , strong S-IR; MM, malignant melanoma.

ranging from faint to (focally) moderate. No S-IR was detected in the suprabasal layers (Fig 1a). Epithelial cells of the adnexa (sebaceous and sweat glands, follicular outer root sheath) were also stained. In particular, at variance with bcl-2, no difference was observed in staining intensity between the different epithelial components of eccrine sweat glands (Rodriguez-Villanueva *et al*, 1995). Whereas follicular epithelium express high levels of bcl-2 in anagen, catagen phase of the cell cycle is associated with apoptosis and characterized by decreased bcl-2 protein (Stenn *et al*, 1994). In this study, survivin was expressed mostly in the follicular outer root sheath. More detailed studies, however, are required to evaluate the potential role of survivin in hair cycling. Fetal skin showed very faint staining in the epidermal basal layer, in agreement with previous observation (Adida *et al*, 1998).

To confirm that survivin is expressed in the basal layer of normal adult human epidermis, keratinocytes derived from newborn foreskin were cultured in serum-free defined medium which selects mostly basal cells. In western blots performed on proteins extracted from these cells the anti-survivin antibody recognized a single band of 16.5 kDa (Fig 1b). A band of the same molecular weight corresponding to survivin was also observed in Jurkat cell lines, used as a positive control (Ambrosini *et al*, 1997). In contrast, no specific bands were immunoblotted by the addition of rabbit IgG under the same experimental conditions.

Expression of survivin in pathologic skin (Table I) S-IR was observed in four of five benign melanocytic lesions, both in the junctional and in the dermal component (Fig 2a). A strong survivin expression was also detected in all Spitz nevi (Fig 2b). Among malignant neoplasms, all melanomas expressed moderate-to-marked S-IR. Immunostaining was stronger in invasive lesions (Fig 2c) and metastatic deposits than in *in situ* melanomas. No difference in staining intensity was observed between melanomas in radial and in vertical growth phase.

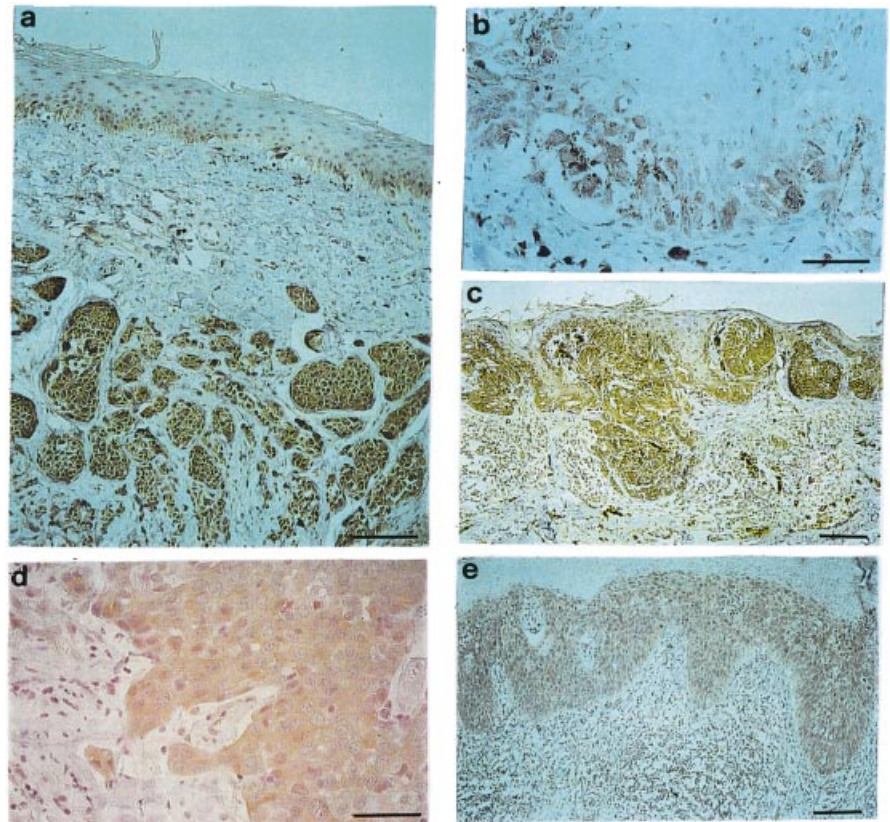


Fig. 2. Expression of survivin in pathologic skin. Intradermal naevus (a), Spitz naevus (b), invasive melanoma (c), SCC (d), Bowen's disease (e). Scale bars: (a, c, e) 100 μ m; (b, d) 50 μ m. Difference in the color of background is due to use of different filters.

Solar and seborrheic keratoses showed a S-IR pattern similar to normal epidermis, survivin expression being confined to the basal layer. S-IR was also observed in all epidermal layers in three of four cases of Bowen's diseases (**Fig 2e**). Six of seven cases of SCC were S-IR positive, staining intensity being stronger in the infiltrative component (**Fig 2d**). On the contrary, no S-IR was observed in all cases of BCC but one, in which a very weak positivity was appreciated. Interestingly, in BCC no S-IR was observed in the epidermis just above the tumor, whereas it was normally present in the basal layer of the normal epidermis adjacent to the lesions.

S-IR in lymphoproliferative diseases (B and T cell lymphomas and pseudolymphomas) was either weak or negative, in accordance with previous observation in low-grade lymphomas, where survivin was undetectable (Ambrosini *et al*, 1997). Merkel cell carcinoma displayed medium-to-focally strong cytoplasmic S-IR.

Colonic adenocarcinoma, used as a positive control, showed strong S-IR in the glandular component. No S-IR was detected in negative controls treated either by preabsorbed antiserum or by omission of the supernatant. No substantial difference in staining intensity was appreciated in specimens treated with the Endogenous Biotin Blocking Kit.

DISCUSSION

Using a combination of immunohistochemical and immunoblotting techniques, we have shown the expression of the novel anti-apoptosis protein survivin in normal human skin. Survivin immunostaining was confined solely to the proliferative basal layer of the epidermis, whereas no staining was detected in the upper layers. This pattern of expression was confirmed by immunoblot assay performed on keratinocytes cultured in a low-calcium medium which selects mostly basal cells. In human epidermis, apoptosis is operational in the suprabasal layers, whereas basal keratinocytes seem to maintain anti-apoptotic defenses (Haake and Polakowska, 1995). Indeed, endonuclease-induced DNA fragmentation is observed in suprabasal, but not in basal keratinocytes (McCall and Cohen, 1991). In addition, bcl-2 protein is expressed exclusively

in the germinative basal cell compartment (Hockenbery *et al*, 1991; Rodriguez-Villanueva *et al*, 1995). Furthermore, it has been shown recently that in basal keratinocytes an autocrine survival system exists sustained by nerve growth factor that protects these cells from apoptosis by inhibiting caspase activation, in a bcl-2-dependent pathway (Pincelli *et al*, 1997; personal communication). Survivin is abundantly expressed in proliferating cells (Li *et al*, 1998). In addition, blocking survivin function results in increased caspase-3 activity, a mechanism involved in cell death (Li *et al*, 1998). Survivin might thus participate in the anti-apoptotic mechanisms in the proliferative basal cell compartment of human epidermis, possibly acting as a direct caspase inhibitor, similarly to other IAP proteins (Deveraux *et al*, 1997).

Interestingly, human skin seems to be the only normal adult tissue expressing survivin (Ambrosini *et al*, 1997, 1998). Human epidermis is characterized by a constant turnover of cells based on the presence in the basal layer of a population of stem cells that retains a high capacity of self-renewal throughout life. One could speculate that survivin, similarly to bcl-2 (Hockenbery *et al*, 1991) protects the viability of regenerating stem keratinocytes, in agreement with what has been proposed in fetal tissues (Adida *et al*, 1998). In this regard, we have recently demonstrated that putative stem keratinocytes express high levels of bcl-2 and are protected from apoptosis (manuscript in preparation).

Whereas survivin was highly expressed in SCC, in agreement with the strong expression of this protein in other carcinomas (Ambrosini *et al*, 1997), no immunostaining was detected in BCC. This is at variance with the expression of bcl-2 that is abundant in BCC and virtually lacking in SCC (Rodriguez-Villanueva *et al*, 1995; Wikonkal *et al*, 1997). Although the significance of this finding requires further studies, it could point to different pathways of survivin and bcl-2 as apoptosis inhibitors. In this regard, it has been shown recently that X-linked IAP inhibit apoptosis in a bcl-2-independent manner (Deveraux *et al*, 1997). Whereas the relationship between bcl-2 and p53 in the pathomechanisms of apoptosis is well defined (Pan *et al*, 1997) and p53 plays a major part in the pathogenesis of SCC (Ziegler *et al*, 1994; Jonason *et al*,

1996), nothing is known on the interplay between p53 and IAP proteins. In SCC, survivin might counteract p53 activity, thus inhibiting the apoptotic process and enhancing tumor development.

In summary, we have shown the expression of the novel anti-apoptosis protein survivin in normal and pathologic skin. These results suggest a possible involvement of survivin and its inhibitory mechanisms in epidermal homeostasis. In addition, these studies point to a putative role of survivin in the development of melanoma and of certain nonmelanoma skin cancers.

This work was supported by grants from the Italian Ministry of University and Scientific Research (MURST) (ex 60%) and by the "Angela Serra" Association for Cancer Research. We gratefully thank Mrs Paola Manni for the skillful technical assistance

REFERENCES

- Adida C, Crotty PL, McGrath J, Berrebi D, Diebold J, Altieri DC: Developmentally regulated expression of the novel cancer anti-apoptosis gene survivin in human and mouse differentiation. *Am J Pathol* 152:43-49, 1998
- Ambrosini G, Adida C, Altieri D: A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma. *Nat Med* 3:917-921, 1997
- Ambrosini G, Adida C, Sirugo G, Altieri DC: Induction of apoptosis and inhibition of cell proliferation by survivin gene targeting. *J Biol Chem* 273:11177-11182, 1998
- Budtz PE. Epidermal homeostasis: a new model that includes apoptosis. In: Tomei LD, Cope FO (eds). *Apoptosis II The Molecular Basis of Apoptosis in Disease*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 1994, pp 165-185
- Cerroni L, Kerl H: Aberrant bcl-2 protein expression provides a possible mechanism of neoplastic cell growth in cutaneous basal-cell carcinoma. *J Cutan Pathol* 21:398-403, 1994
- Clem RJ, Miller LK. Induction and inhibition of apoptosis by insect viruses. In: Tomei LD, Cope FO (eds). *Apoptosis II The Molecular Basis of Apoptosis in Disease*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 1994, pp 89-110
- Deveraux QL, Takahashi R, Salvesen GS, Reed JC: X-linked IAP is a direct inhibitor of cell-death proteases. *Nature* 388:300-304, 1997
- Duckett CS, Nava VE, Gedrich RW, et al: A conserved family of cellular genes related to the baculovirus *iap* gene and encoding apoptosis inhibitors. *EMBO J* 15:2685-2694, 1996
- Fanidi A, Harrington EA, Evan GI: Cooperative interaction between *c-myc* and *bcl-2* protooncogenes. *Nature* 359:554-556, 1992
- Haake AR, Polakowska RR: UV-induced apoptosis in skin equivalents: inhibition by phorbol ester and *bcl-2* expression. *Cell Death Differentiation* 2:183-193, 1995
- Hockenbery DM, Zutter M, Hickey W, Nahm M, Korsmeyer SJ: BCL2 protein is topographically restricted in tissues characterized by apoptotic cell death. *Proc Natl Acad Sci USA* 88:6961-6965, 1991
- Jonason AS, Kunal S, Price GJ, et al: Frequent clones of p53-mutated keratinocytes in normal human skin. *Proc Natl Acad Sci* 93:14025-14029, 1996
- Kanter-Lewenson L, Hedblad MA, Wejde J, Larsson O: Immunohistochemical markers for distinguishing Spitz nevi from malignant melanomas. *Mod Pathol* 10:917-920, 1997
- Kroemer G: The proto-oncogene *Bcl-2* and its role in regulating apoptosis. *Nat Med* 3:614-620, 1997
- LeBrun DP, Warnke RA, Cleary ML: Expression of *bcl-2* in fetal tissues suggest a role in morphogenesis. *Am J Pathol* 142:743-753, 1993
- Li F, Ambrosini G, Chu EY, Plescia J, Tognin S, Marchisio PC, Altieri DC: Control of apoptosis and mitotic spindle checkpoint by survivin. *Nature* 396:580-584, 1998
- Liston P, Roy N, Tamai K, et al: Suppression of apoptosis in mammalian cells by NAIP and a related family of IAP genes. *Nature* 379:349-353, 1996
- Lovering R, Hanson IM, Borden KL, et al: Identification and preliminary characterization of a protein motif related to the zinc finger. *Proc Natl Acad Sci USA* 90:2112-2116, 1993
- McCall CA, Cohen JJ: Programmed cell death in terminally differentiating keratinocytes: role of endogenous endonuclease. *J Invest Dermatol* 97:111-114, 1991
- Morales-Ducret CR, van de Rijn M, LeBrun DP, Smoller BR: *bcl-2* expression in primary malignancies of the skin. *Arch Dermatol* 131:909-912, 1995
- Norris DA, Middleton MH, Whang K, et al: Human keratinocytes maintain reversible anti-apoptotic defenses in vivo and in vitro. *Apoptosis* 2:136-148, 1997
- Pan H, Van Yin C, Dyke T: Apoptosis and cancer mechanisms. *Cancer Surv* 29:305-327, 1997
- Pincelli C, Haake A, Benassi L, et al: Autocrine nerve growth factor protects human keratinocytes from apoptosis through its high affinity receptor (trk): a role for *bcl-2*. *J Invest Dermatol* 109:757-764, 1997
- Polakowska RR, Haake AR: Apoptosis: the skin from a new perspective. *Cell Death Differentiation* 1:19-31, 1994
- Polakowska RR, Piacentini M, Bartlett R, Goldsmith LA, Haake AR: Apoptosis in human skin development: morphogenesis, periderm, and human stem cells. *Dev Dynamics* 199:176-188, 1994
- Raff M: Social controls on cell survival and cell death. *Nature* 356:397-400, 1992
- Rodriguez-Villanueva J, Colome MI, Brisbay S, McDonnell TJ: The expression and localization of *bcl-2* protein in normal skin and in non-melanoma skin cancers. *Pathol Res Pract* 191:391-398, 1995
- Stenn KS, Lawrence L, Veis D, Korsmeyer S, Seiberg M: Expression of the *bcl-2* protooncogene in the cycling adult mouse hair follicle. *J Invest Dermatol* 103:107-111, 1994
- Thompson CB: Apoptosis in the pathogenesis and treatment of disease. *Science* 267:1456-1462, 1995
- Uren AG, Pakusch M, Hawkins CJ, Puls KL, Vaux DL: Cloning and expression of apoptosis inhibitory protein homologs that function to inhibit apoptosis and/or bind tumor necrosis factor receptor-associated factors. *Proc Natl Acad Sci USA* 93:4974-4978, 1996
- Wikonkal NM, van Berg RJW, Haselen CW, et al: *bcl-2* vs p53 protein expression and apoptotic rate in human nonmelanoma skin cancers. *Arch Dermatol* 133:599-602, 1997
- Yaar M, Eller MS, DiBenedetto P, et al: The trk family of receptors mediates nerve growth factor and neurotrophin-3 effects in melanocytes. *J Clin Invest* 94:1550-1562, 1994
- Zhai S, Yaar M, Doyle SM, Gilchrist BA: Nerve growth factor rescues pigment cells from ultraviolet-induced apoptosis by upregulating BCL-2 levels. *Exp Cell Res* 224:335-343, 1996
- Ziegler A, Jonason AS, Leffel DJ, et al: Sunburn and p53 in the onset of skin cancer. *Nature* 372:773-776, 1994