Association of Tumor Necrosis Factor-α Gene Expression and Apoptotic Cell Death with Regression of Shope Papillomas

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The objective of this study was to test the hypothesis that spontaneous regression of Shope papillomas involves tumor necrosis factor-α and apoptotic cell death of the papilloma cells. *In situ* hybridization using RNA probes of rabbit tumor necrosis factor-α revealed tumor necrosis factor-α mRNA in most of the numerous mononuclear cells infiltrating the upper dermis of regressing papillomas and at the dermoepidermal junction. Such cells in progressing papillomas were much fewer in number and were located in the deeper dermis. *In situ* terminal deoxynucleotidyl transferase assay demonstrated DNA strand breaks in many scattered epidermal keratinocytes of regressing papillomas but in only a few thin layers just beneath the horny layer in progressing papillomas. Electron microscopy demonstrated that regressing papillomas contained many apoptotic bodies and keratinocytes showing apoptotic changes such as chromatin condensation, degradation of condensed nuclei, surface protuberances, and a filamentous degeneration, as well as infiltrating lymphocytes and macrophages. We propose that tumor necrosis factor-α produced by infiltrating mononuclear cells probably plays a role in the papilloma regression. ***Key words: cottontail rabbit papillomavirus/DNA strand break/electron microscopy/in situ hybridization. J Invest Dermatol 104:526–529, 1995***

Viral warts caused by human papillomaviruses and cottontail rabbit papillomaviruses occasionally undergo spontaneous regression, accompanied by massive infiltrates of mononuclear cells [1]. This phenomenon has been a subject of interest for both pathologists and dermatologists because it represents an example of naturally occurring tumor immunity [2,3].

There are two major areas of interest in studying papilloma regression: the nature of the infiltrates and the mechanism(s) by which massive cell loss occurs to achieve complete disappearance of papillomas. It has been demonstrated that the cells infiltrating regressing warts are mainly lymphocytes and macrophages [4,5] and that the lymphocytes consist mainly of T cells [6], which express either CD4 or CD8 [7]. These findings suggest a key role of cell-mediated immunity in this process. On the other hand, early electron microscopic observations have demonstrated apoptosis or dyskeratosis in regressing plane warts [4,5]. It was also revealed that keratinocytes of regressing papillomas showed reduced proliferative activity compared with those of progressing warts [8,9]. These data could explain the reduction of papilloma volume during regression.

Although these data suggest that the infiltrating cells may play a role in inducing cell death or inhibiting growth of the infected keratinocytes, the exact nature of the factors that mediate these cytotoxic or cytostatic effects remains obscure. We tested the hypothesis that the infiltrating cells produce tumor necrosis factor-α (TNF-α). This cytokine has recently been found to induce apoptosis of sensitive target cells [10,11] and to be produced by various cell types, including T lymphocytes and macrophages [12]. Apoptosis is also involved in cell-mediated immunity [11].

In this report, we describe the detection of TNF-α mRNA by *in situ* hybridization (ISH), DNA strand breaks by *in situ* terminal deoxynucleotidyl transferase assays (ISTdT), and morphologic evidence of apoptosis by electron microscopy. We suggest that TNF-α plays a role in regression of papillomas by inducing apoptosis of papilloma cells.

MATERIALS AND METHODS

Animals Ten New Zealand White rabbits were purchased from Gingrich Animal Supply (Fredericksburg, PA) and inoculated with a cottontail rabbit papillomavirus stock [8] at six sites per rabbit on the dorsal skin. Three rabbits with regressing papillomas were obtained as described previously [9].

Tissue Preparation Regressing and progressing papillomas were excised and divided into several parts. The specimens were fixed in buffered formalin and processed routinely for light microscopy, ISH, and ISTdT. Some specimens of regressing papillomas were fixed in 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) and 5% sucrose, post-fixed in 1% osmium tetroxide in the same buffer, and embedded in an epoxy resin. Archival paraffin blocks derived from the previous experiments [6,9] were also used.

RNA Probes A Pst I fragment (1.2 kilobase pairs) of rabbit TNF-α cDNA that encompassed the entire coding sequence was obtained from Asahi Chemical Industry Co. (Fuji-shi, Japan) [13]. The DNA was subcloned into pGEM3zf (−) vector (Promega Corp, Madison, WI) using JM109 competent cells according to the manufacturer's protocol. Digoxigenin-labeled antisense and sense RNA strands were obtained in full length by *in vitro*

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Abbreviations: ISH, *in situ* hybridization; ISTdT, *in situ* terminal deoxynucleotidyl transferase assay.

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transcription using a Genius 4 RNA labeling kit (Boehringer Mannheim Corp., Indianapolis, IN) and then hydrolyzed by partial alkaline hydrolysis into approximately 150-base pair fragments [14].

ISH Using Digoxigenin-Labeled RNA Probes ISH was performed as described previously [15], with some modifications. Deparaffinized sections (from seven regressing and five progressing papillomas) were treated with proteinase K as described previously [16], acetylated with acetic anhydride, dehydrated, and air dried. The sections were prehybridized in 50% formamide, 2 × standard saline citrate (SSC), 4 × Denhardt's solution (50 × solution consists of 1% Ficoll, 1% polyvinylpyrrolidone, and 1% bovine serum albumin), 1 mg/ml salmon testicular DNA, 1 mg/ml yeast tRNA, and 0.2 mg/ml poly A, at 45°C for 1 h. The sections were then hybridized in 50% formamide, 10% dextran sulfate, 2 × SSC, 1 × Denhardt's solution, 0.5 mg/ml salmon testicular DNA, 0.5 mg/ml yeast tRNA, 0.1 mg/ml poly A, and 2–3 ng/μl digoxigenin-labeled RNA under siliconized coverslips at 45°C overnight. After the coverslips were removed in 2 × SSC, the sections were treated with 40 μg/ml RNase A and 2 μg/ml RNase T_1 in 2 × SSC at 52°C for 1 h, and in 2 × and 0.1 × SSC each for 1 h at room temperature. The sections were incubated with 0.5% blocking reagent (Boehringer Mannheim) in 50 mM Tris-HCl (pH 7.5) and 150 mM NaCl (Tris-buffered saline) for 30 min, then with antidigoxigenin antibody coupled to alkaline phosphatase (Boehringer Mannheim), diluted 1:1000 in the blocking solution, for 1 h. The sections were rinsed in Tris-buffered saline containing 0.1% Tween 20 and 0.1% Triton X-100 and then in 100 mM Tris-HCl (pH 9.5), 100 mM NaCl, and 50 mM $MgCl_2$ (substrate buffer). Color was developed by incubating the sections with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate in the substrate buffer for 6 h or overnight. Finally, the sections were counterstained with nuclear fast red and mounted with an aqueous mounting reagent. Negative controls were performed in two different ways: first by using the sense probe, and second by pretreating the sections with RNase (0.1 mg/ml RNase A and 5 μg/ml RNase T_1 in 10 mM Tris-HCl [pH 7.5] and 15 mM NaCl at 37°C for 1 h before the prehybridization step).

ISTdT These assays were performed as described previously [17], with modifications. Briefly, deparaffinized sections were incubated with 50 μl of reaction mixture, which contained 100 mM sodium cacodylate (pH 7.0), 1 mM $CoCl_2$, 0.1 mM dithiothreitol, 50 μg/ml bovine serum albumin, 0.1 μl DIG DNA labeling mixture (Boehringer Mannheim), and 2.5 U terminal transferase (Boehringer Mannheim), at 37°C for 30 min. The incorporated digoxigenin-labeled reagents were detected as described above. Positive controls were performed by introducing nicks into DNA in the sections by incubating the sections with 50–100 ng/ml DNase I in 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM dithiothreitol, and 50 μg/ml bovine serum albumin before the labeling step. Negative controls were performed by omitting terminal deoxynucleotidyl transferase from the reaction.

Electron Microscopy Ultrathin sections (80–100 nm thick) were cut from the epoxy blocks, stained with uranyl acetate and lead citrate, and observed with a Phillips 400 electron microscope at 60 kV.

RESULTS

ISH for TNF-α mRNA Five freshly prepared and seven archival sections showed consistent results (seven regressing and five progressing papillomas). In regressing papillomas, numerous mononuclear cells infiltrated the dermis, mainly from the upper dermis, showing fibrosis, to the dermoepidermal junction. Most of the infiltrates showed staining with the antisense probe for TNF-α mRNA exclusively in their cytoplasm **(Fig 1*a*)**. These stained cells were of two types. Cells possessing a thin rim of cytoplasm, which were regarded as lymphocytes, were located mainly in the upper dermis **(Fig 1*b*)**. In the mid- and lower dermis, the cells became more sparse and chiefly contained abundant elongated or polygonal cytoplasm **(Fig 1*c*)**, and represented macrophages or histiocytes. On the other hand, progressing papillomas contained only a few scattered cells showing staining; such cells were usually located deeper in the dermis and were not found at the dermoepidermal junction **(Fig 1*d*)**. Although we observed weak staining of keratinocytes in both regressing and progressing papillomas **(Fig 1*b,d*)**, we regarded it as nonspecific because similar staining was also found in the sections stained with the sense probe **(Fig 1*e*)**. Negative controls using the sense probe **(Fig 1*e*)** or RNase pretreatment (not shown) showed little or no staining in the dermis of both regressing and progressing papillomas.

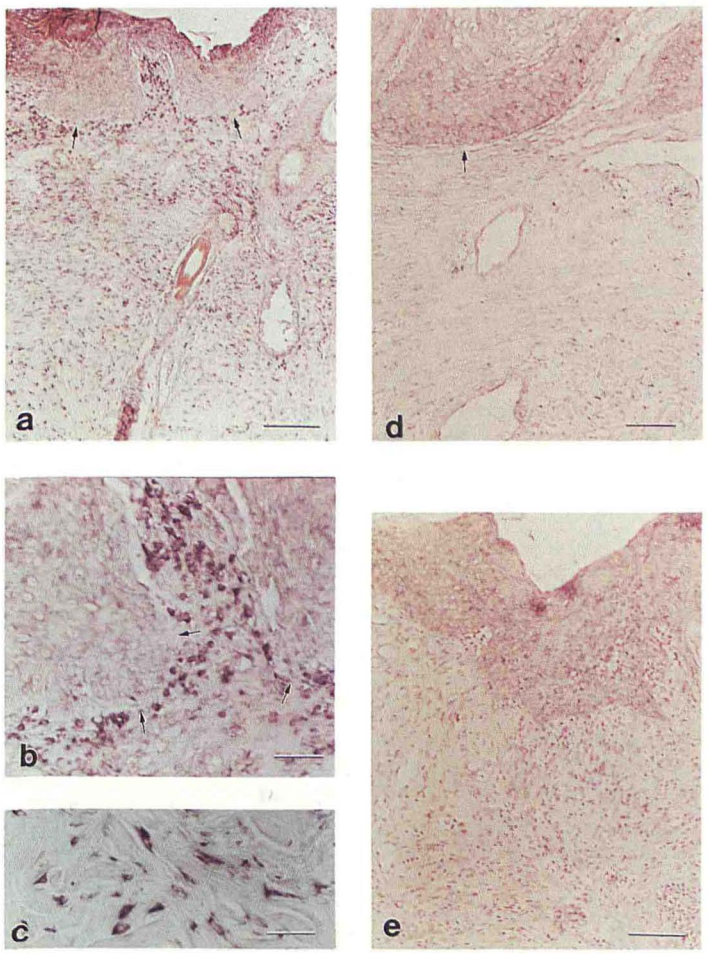

Figure 1. ISH with digoxigenin-labeled TNF-α RNA. *Arrows* indicate the dermoepidermal junction. *a*) Regressing papilloma hybridized with the antisense probe. There are numerous stained cells infiltrating the dermis. *b*) Epidermis and upper dermis of (*a*). Most cells possess a thin rim of cytoplasm stained by ISH. *c*) Deeper dermis of (*a*). Stained cells have elongated or polygonal cytoplasm. *d*) Progressing papilloma hybridized with the antisense probe. Cells stained with ISH are not observed in the upper dermis. *e*) Regressing papilloma hybridized with the sense probe. Weak cytoplasmic staining of keratinocytes is seen. *Bars*: *a*, 200 μm; *b* and *c*, 50 μm; *d* and *e*, 100 μm.

ISTdT In regressing papillomas, many scattered keratinocytes in the epidermis showed strong nuclear staining by ISTdT **(Fig 2*a*)**, whereas in progressing papillomas, such nuclear staining was demonstrated only in a few thin layers just beneath or in the horny layer. Here keratinocytes showed vacuolization, i.e., viral cytopathic effects of cottontail rabbit papillomavirus **(Fig 2*b*)**. In either case, dermal cells showed no nuclear staining. Positive control DNase-I–treated sections showed strong nuclear staining even in cells infiltrating the dermis **(Fig 2*c*)**. There was no staining in negative control sections, in which terminal deoxynucleotidyl transferase was omitted from the reaction **(Fig 2*d*)**.

Electron Microscopy Electron microscopy of regressing papillomas revealed that the nuclei of basal and suprabasal keratinocytes showed chromatin condensation along the nuclear envelope, and some of them were degraded into fragments **(Fig 3*a*)**. Such cells possessed relatively light cytoplasm containing loosely packed wavy filaments, scattered mitochondria, ribosomes, and a few small vacuoles. They still demonstrated distinct desmosomes, although the cytoplasm appeared to detach from the neighboring cells and the basal lamina **(Fig 3*a*)**. In the upper portion of the epidermis, a keratinocyte with nuclear and cytoplasmic condensation showed surface protuberances **(Fig 3*b*)**. Cellular debris containing a nuclear

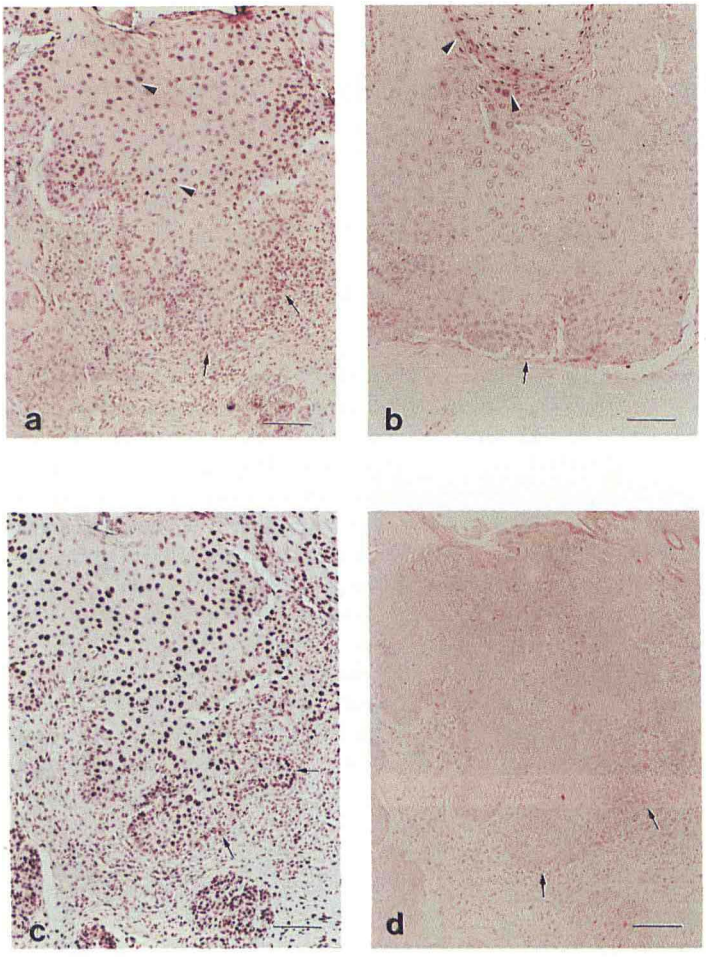

Figure 2. ISTdT. *Arrows* indicate the dermoepidermal junction. *a*) Regressing papilloma. A number of scattered epidermal keratinocytes show nuclear staining (*arrowheads*). *b*) Progressing papilloma. Stained nuclei are seen in one or a few layers just beneath and in the horny layer (*arrowheads*). *c*) Positive control (regressing papilloma) in which DNase I pretreatment was performed. Both epidermal and dermal cells are stained. *d*) Negative control (regressing papilloma) in which the labeling enzyme was omitted from the reaction. There is no staining. *Bars*, 100 μm.

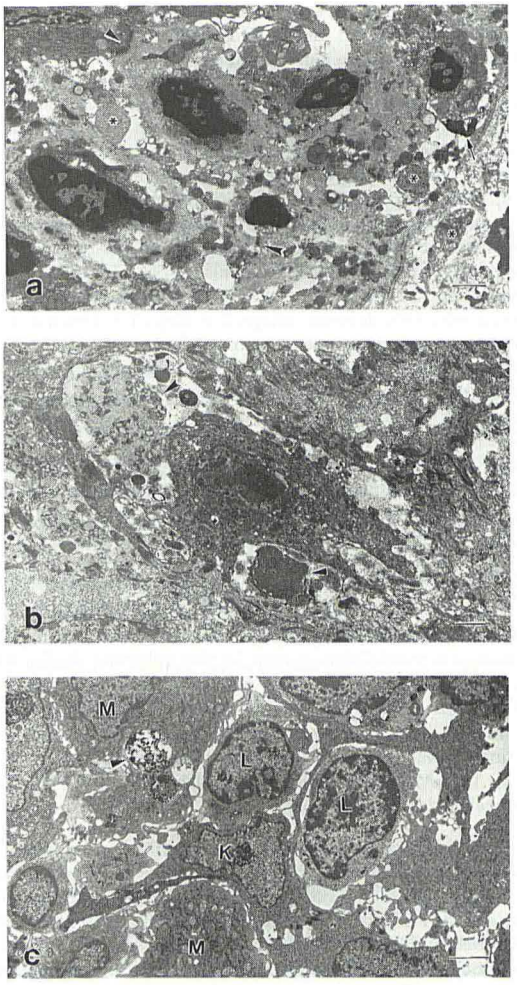

Figure 3. Apoptotic changes of keratinocytes in a regressing papilloma. *a*) Basal and suprabasal keratinocytes possess a nucleus with chromatin condensed along the nuclear envelope. Degradation of the condensed nucleus is also seen (*arrow*). Some of these cells still retain desmosomes (*arrowheads*). Apoptotic bodies (*asterisks*) can be seen in the intercellular space or in the upper dermis. *b*) A keratinocyte with nuclear and cytoplasmic condensation and surface protuberances is seen in the upper portion of the epidermis. Apoptotic bodies (*arrowheads*) are also seen in the intercellular space. *c*) Lymphocytes (L) and macrophages (M) are seen in contact with a keratinocyte (K). Phagocytosed apoptotic bodies (*arrowhead*) are seen in the cytoplasm of a macrophage. *Bars*: *a* and *b*, 1 μm; *c*, 2 μm.

remnant composed of condensed chromatin or other cell organelles (representing apoptotic bodies) was found in the intercellular space, in the upper dermis **(Fig 3*a,b*)**, or in the cytoplasm of macrophages and keratinocytes in the epidermis **(Fig 3*c*)**. An occasional cell just beneath the granular layer contained a central mass, closely packed bundles of tonofilaments, which were compressed toward the periphery of the cytoplasm, and numerous vacuoles **(Fig 4*a*)**. The central mass comprised numerous wavy filaments, which were adjacent to the peripheral tonofibrils, and many spherical or oval bodies representing remnants of cytoplasmic organelles **(Fig 4*b*)**. The infiltrating cells consisted mainly of lymphocytes, with a round or indented nucleus and scant to moderately abundant cytoplasm, and of macrophages **(Fig 3*c*)**. Cells with the features of large granular lymphocytes were not encountered.

DISCUSSION

We detected TNF-α mRNA in most of the infiltrating cells of the regressing papillomas. The majority of these cells were concentrated in an area from the dermoepidermal junction to the upper dermis. In contrast, the number of such cells in progressing papillomas was much smaller and the cells infiltrated only the deeper dermis; none were seen at the dermoepidermal junction. This difference in the infiltrates is consistent with previous results obtained by morphometric analysis [6]. These data indicate that expression of the TNF-α gene is associated with the regression phenomenon.

ISTdT has been used to detect DNA strand breaks as positive staining [17,18]. This assay revealed distinct staining patterns between progressing and regressing papillomas. In progressing papillomas, labeling was mostly confined to the area just beneath the horny layer, corresponding to the granular layer. On the other hand, regressing papillomas showed numerous scattered, labeled nuclei throughout the epidermis, including the lower part of the epidermis. Staining patterns similar to that of progressing papillomas have been described in normal epidermis [18]. Furthermore, induction of DNA strand breaks related to terminal differentiation of keratinocytes has been suggested. In fractionated keratinocytes from normal skin, the fraction of granular keratinocytes, but not basal keratinocytes, has shown DNA fragmentation characteristic of apoptosis [19]. These observations therefore suggest that progressing papillomas, unlike regressing papillomas, undergo DNA strand breaks in a way similar to that of normal skin. It is also possible that such nuclear alterations in progressing papillomas may be overlapped by those associated with productive infection of cottontail

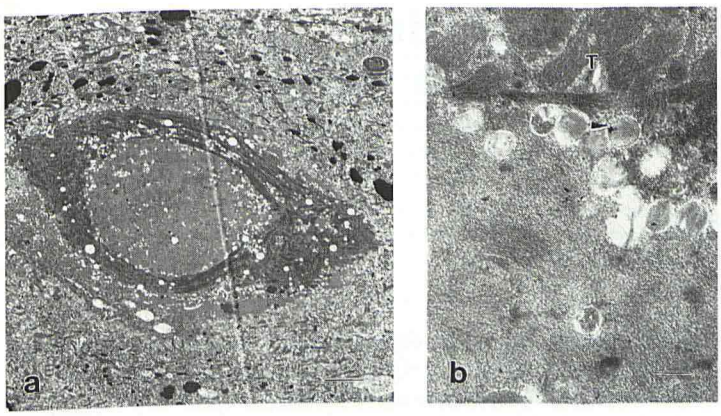

Figure 4. Keratinocyte showing filamentous degeneration in a regressing papilloma. *a*) A keratinocyte adjacent to the granular layer contains a central mass, numerous vacuoles, and tonofilaments, which are compressed toward the periphery of the cytoplasm. *b*) The central mass is composed of wavy filaments in conjunction with tonofilaments (T) and spherical bodies (*arrowhead*), representing remnants of cytoplasmic organelles. *Bars*: *a*, 2 μm; *b*, 0.2 μm.

rabbit papillomavirus, because vegetative DNA replication of papillomaviruses also becomes prominent at the granular layer [16,20]. On the other hand, the close association between cells that express TNF-α and regressing papillomas suggests that this cytokine is responsible for the aberrant pattern of DNA strand breaks observed in regressing papillomas, because TNF-α is known to induce DNA strand breaks associated with apoptosis [10,11].

Electron microscopic examination of regressing papillomas demonstrated morphologic alterations compatible with those observed in a variety of conditions showing apoptosis [11]. These include chromatin condensation along the nuclear envelope of keratinocytes, degradation of such altered nuclei, surface protuberances of keratinocytes showing nuclear and cytoplasmic condensation, and a number of apoptotic bodies engulfed by macrophages or neighboring keratinocytes within regressing papillomas. In addition, we observed a filamentous degeneration, a special form of keratinocyte alteration that is also believed to represent apoptosis [21]. Furthermore, the observation of some of these alterations even in the basal layer supports the aberrant staining pattern of ISTdT found in regressing papillomas. These results from both ISTdT and electron microscopy strongly suggest that apoptosis serves as one component of the mechanism for papilloma regression.

Electron microscopy also revealed inflammatory cells in the epidermis of regressing papillomas. However, ISH could not demonstrate any exocytotic cells showing cytoplasmic staining in the epidermis, although massive mononuclear cells in the upper dermis were stained. A possible explanation for this discrepancy is that these inflammatory cells expressing TNF-α may quickly release the cytokine during the exocytotic process.

TNF-α is a cytokine produced by various cell types consisting of macrophages and T lymphocytes [12]. We could identify at least two cell types by ISH: cells with a thin rim of cytoplasm and those with abundant cytoplasm, which corresponded to lymphocytes and to macrophages or histiocytes, respectively, observed with electron microscopy. Because keratinocytes are also revealed to produce TNF-α after certain stimuli [22], it is possible that they may be involved in the production of TNF-α associated with papilloma regression. In the present study, however, we excluded the participation of keratinocytes in producing TNF-α in this process because we observed no definite staining of keratinocytes using ISH.

The cellular constituents observed in our experiments are compatible with early electron microscopic studies [4,5]. Other studies have characterized further the surface phenotypes of infiltrates in regressing Shope papillomas or plane warts. The majority of the infiltrating lymphocytes were T lymphocytes [6] expressing either CD4 or CD8 [7]. However, the immune functions of these cells were not determined in these experiments. Further investigations will be required to correlate TNF-α gene expression with surface phenotype.

In this report, we demonstrated an association of TNF-α gene expression and apoptosis with regression of Shope papillomas, although causative effects of the cytokine remain to be demonstrated. In addition, it is possible that other pathways, such as perforin-granzyme and Fas protein (CD95), are also involved in apoptosis associated with papilloma regression. We suggest that TNF-α is at least one of the factors involved in this phenomenon. Further study of the involvement of this cytokine in papilloma regression will provide us with a more complete understanding.

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