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Shope Papilloma Cell and Leukocyte Proliferation in Regressing and Progressing Lesions

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Lesions generated by infection with cottontail rabbit papillomavirus frequently undergo spontaneous regression. The purpose of this immunohistochemical study was to compare leukocyte and papilloma cell proliferation in progressing and regressing papillomas and to test the hypothesis that regression was associated with an inhibition of papilloma cell proliferation. The monoclonal antibodies (MAbs) MAb-019 (specific for DNA/bromodeoxyuridine [BrdU] complexes), Ki-67 (specific for actively proliferating cells), L11/135 (specific for rabbit T cells), and 2C4 (specific for rabbit class II antigen) were used for this purpose. In progressing papillomas, there were few leukocytes (<1%) in the dermis that stained with MAb-019 and Ki-67, whereas these antibodies stained 4.5% and 6.8% of the intraepidermal leukocytes, respectively. Regressing papillomas contained conspicuous leukocytic infiltrates in the dermis, of which 76.9% were L11/135-positive T cells. However, few intradermal leukocytes (<3%) stained positively with MAb-019 and Ki-67 MAbs, despite expressing rabbit class II antigen. The epidermis of regressing papillomas contained a higher percentage of MAb-019- and Ki-67-positive leukocytes than the epidermis of progressing papillomas. Intraepidermal leukocytes in progressing and regressing papillomas consisted mainly of T cells stained by L11/135. It appeared that many dermal leukocytes (mainly T cells) form a non-cycling T cell population in both progressing and regressing papillomas, whereas intraepidermal T cells in regressing papillomas were effectively activated and represented a cycling T cell population. MAb-019 and Ki-67 MAbs demonstrated similar staining patterns in papilloma and normal tissues. However, in both progressing and regressing papillomas, the Ki-67 MAb usually stained a larger percentage of cells than the MAb-019 MAb. MAb-019 and Ki-67 MAbs showed a homogenous distribution of positive cells from basal layer to the upper layer in progressing papillomas. On the other hand, in regressing papillomas, cell staining with the two antibodies was concentrated in the basal and lower layers, but not in the upper layers. This result indicates that cell proliferation in the upper epidermal layers is suppressed in regressing papillomas. Our present data show that intraepidermal T- cell activation and suppression of tumor proliferation might play a crucial role in papilloma regression. (Am J Pathol 1993, 142:489–496)

It is widely accepted that human papillomaviruses are causative agents associated with a variety of cutaneous and mucosal squamous epithelial lesions in humans.[1–3] Some of the tumors induced by human papillomaviruses spontaneously regress after the sudden occurrence of inflammation. In addition, Shope papillomas induced by the cottontail rabbit papillomavirus (CRPV) show similar spontaneous regression. Shope rabbit papillomas have long been a useful animal model for human neoplasia and human papillomavirus infection.[4,5] Spontaneously regressing lesions are infiltrated by a large number of leukocytes, mainly lymphocytes, suggesting that cell-mediated immunity plays an important role in the rejection of papillomas.[4,6,7] Previously, our data have shown that most of the infiltrating mononuclear cells in regressing rabbit papillomas were T cells (about 70%) and expressed rabbit class II antigen.[8] However, the role of those infiltrating lymphocytes in tumor regression is not well known. We tested the hypothesis that regression was associated with an

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inhibition of papilloma cell proliferation. To examine this, three monoclonal antibodies (MAb-019, Ki-67, L11/135) were used in immunohistochemical analysis of rabbit papillomas. Previously, proliferation of tumors was measured with the use of tritiated thymidine and autoradiography.[7,9,10] However, the use of tritiated thymidine was strictly limited because of isotope contamination hazards. To overcome this problem, the use of the nonradioactive thymidine analogue bromodeoxyuridine (BrdU) and a monoclonal antibody against BrdU (MAb-019) were developed.[11–13] The Ki-67 MAb[14] is an alternative that recognizes a human nuclear antigen that is expressed by cells in all phases of the cell cycle, but not by resting or differentiated cells.[15] The Ki-67 MAb is now widely used as a method for assessment of the growth fraction in human tumors.[16,17] Recently the Ki-67 antigen has been characterized as a nonhistone nuclear protein with a molecular weight of 395 kd and 345 kd.[18] In addition, it has been shown that this MAb also reacts with rabbit tissues.[19] The L11/135 MAb was used to identify all T lymphocytes.[20] In this study, we tested the hypothesis that proliferation of papilloma cells and leukocytes might differ between progressing and regressing papillomas.

Materials and Methods

Animals

A total of 20 New Zealand White rabbits was purchased from Gingrich Animal Supply, Fredricksburg, PA. CRPV was obtained from wild cottontail papilloma extracts.[7] All rabbits were inoculated at four sites with a 10^{-2} dilution of viral stocks onto an abraded area (1 × 1 cm) on the dorsal skin. Papillomas were measured once or twice per week. Rabbits were identified as regressors by the complete involution of at least one papilloma site with a concomitant reduction in papilloma volume at all remaining sites. While a total of 6 regressor rabbits were obtained, rapid disappearance of all papillomas on 3 of these excluded them from analysis. At least 1 papilloma from each of the 3 remaining regressors as well as 2 to 3 papillomas from each progressor rabbit were analyzed. Three regressor rabbits and 6 progressor rabbits were injected through the marginal ear vein with a total dose of 50 mg/kg BrdU in a 0.1 mol/L carbonate buffer 3 hours before sacrifice.

Tissue Preparation

Three regressing papillomas from three regressor rabbits were used for Ki-67 and L11/135 MAb staining, and four regressing papillomas from three regressor rabbits were examined using MAb-019. Papillomas from six progressor rabbits were stained with MAb-019 (17 sites), Ki-67 (15 sites), and L11/135 (16 sites) MAbs. All progressing and regressing papillomas were divided into two parts and either fixed in 10% neutral-buffered formalin or embedded in OCT compound (Miles Inc., Elkhart, IN) and snap-frozen in liquid nitrogen. Frozen tissue blocks were stored at –70 C until use. Formalin-fixed tissues, paraffin-embedded, were used for routine hematoxylin and eosin staining and immunohistochemical staining with the MAb-019 MAb.

Monoclonal Antibodies

L11/135 hybridoma cells (recognizing all rabbit T lymphocytes)[20] were obtained from the American Type Culture Collection, Rockville, MD (catalog no. TIB188) and cultured in RPMI 1640 medium (1% fetal calf serum), 200 mmol/L glutamine, 25 mmol/L *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, 100 U streptomycin and penicillin. Four- to five-day culture supernatants were used directly as a 1:4 dilution with 4% bovine serum albumin in phosphate-buffered saline. Two mouse MAbs, Ki-67 (specific for nuclear antigen present only in proliferating cells)[14] and MAb-019 (specific for BrdU-incorporated into DNA), were purchased from Dakopatts (Santa Barbara, CA) and Chemicon (Tewecula, CA), respectively.

Staining Procedures

Six-micron-thick cryostat sections of snap-frozen tissues were placed on silane-coated slides, air-dried at room temperature, then fixed in cold acetone for 10 minutes. Alternatively, 4-μ-thick formalin-fixed, paraffin sections were dewaxed in xylene and rehydrated with a graded series of ethanol. Subsequently, the slides were put in 0.3% peroxidase blocking solution for 10 minutes at room temperature. Afterward, all slides were incubated with nonimmune 10% rabbit serum to block nonspecific Fc receptor binding and then treated with appropriate primary antibodies, MAb-019 (at a dilution of 1:20), Ki-67 (at 1:16), and L11/135 (at 1:4), for 30 minutes at 37 C. After incubation, the slides were rinsed and stained by an avidin-biotin complex staining method of the Histostain-sp kit for mouse MAbs according to the manufacturer's protocol (Zymed Laboratories, South San Francisco, CA). This staining procedure was performed by a Code-On Immunostainer (Fisher Scientific, Pittsburgh, PA).

Morphometry

Quantification of positive cells was achieved by microscopic observation via superimposition of a 0.25 × 0.25 mm ocular grid on the microscopy field.[7] The center of the grid was placed on the epidermal basement membrane so that the grid extended 125 μ above and 125 μ below the membrane. The grid was divided into 6 ranks in the vertical dimension: ranks 1, 2, and 3 were in the epidermis, and ranks 4, 5, and 6 were in the dermis. Ranks 1, 2, 5, and 6 corresponded to strips of 50 μ, and ranks 3 and 4 corresponded to strips of 25 μ in the vertical dimension. For example, rank 1 extended from 125 μ to 75 μ from the basement membrane, rank 2 extended from 75 μ to 25 μ from the basement membrane, and rank 3 extended from the basement membrane to 25 μ above, etc. Each rank corresponded to 250 μ in horizontal dimension (Figure 1). Within each rank, all targeted cells were counted and the grid was moved laterally, in non-overlapping fields, from the marginal to central portion of the section. At least three fields were counted in each section. Enumeration of positive cells was carried out at a magnification of ×400, and Student's *t*-test was used for statistical analysis.

Results

Analysis of MAb-019 and Ki-67 MAb-Positive Keratinocytes in Progressing and Regressing Papillomas

As shown in Figures 2 and 3 in both progressing and regressing papillomas, MAb-019 and Ki-67 revealed very similar staining patterns. In progressing papillomas, these two antibodies demonstrated relatively homogenous staining among the epidermal ranks. For example, 20.9% of the cells in rank 1 were MAb-019-positive, whereas 28.6% were positive in rank 3. On the other hand, 68.4% of the tumor cells in rank 1 were Ki-67-positive, and 49.0% were positive in rank

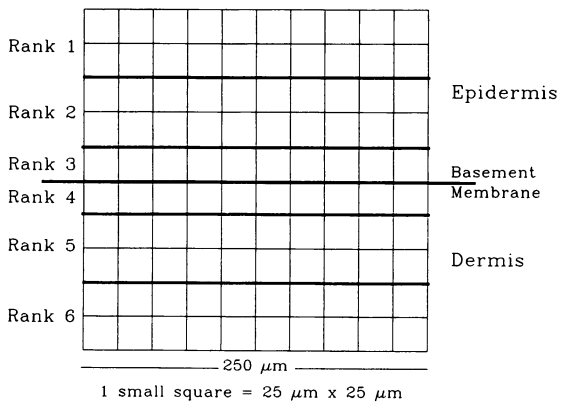

Figure 1. *Quantification of positive cells was achieved by superimposition of a 0.25 × 0.25 mm ocular grid on the microscopy field. The center of the grid was placed on the epidermal basement membrane so that in the vertical dimension the grid extended 125 μ above and 125 μ below the membrane. The grid was divided into 6 ranks in the vertical dimension; ranks 1, 2, and 3 were in the epidermis, whereas ranks 4, 5, and 6 were in the dermis.*

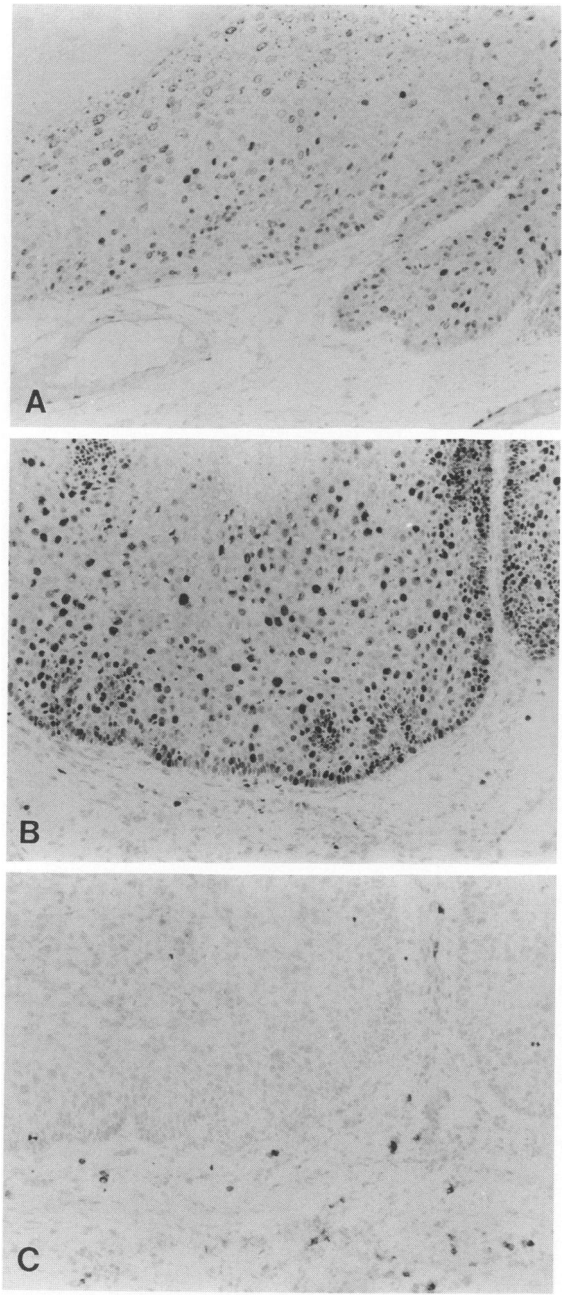

Figure 2. *Distribution of proliferating cells in progressing papillomas.* A, B, C: *MAb-019 (anti-BrdU), Ki-67, and L11/135 (T cell), respectively. Progressing papillomas show high and equal percentages of MAb-019* (A)- *and Ki-67* (B)*-positive papilloma cells extending from the basal layer to the upper layer in the epidermis. Few infiltrating leukocytes can be seen in either the dermis or epidermis of the section. MAb-019 and Ki-67 labeled few leukocytes in the dermis whereas a low, but higher percentage, were labeled within the epidermis. These intraepidermal leukocytes consist mostly of L11/135-positive T cells* (C). *Magnification, ×130.*

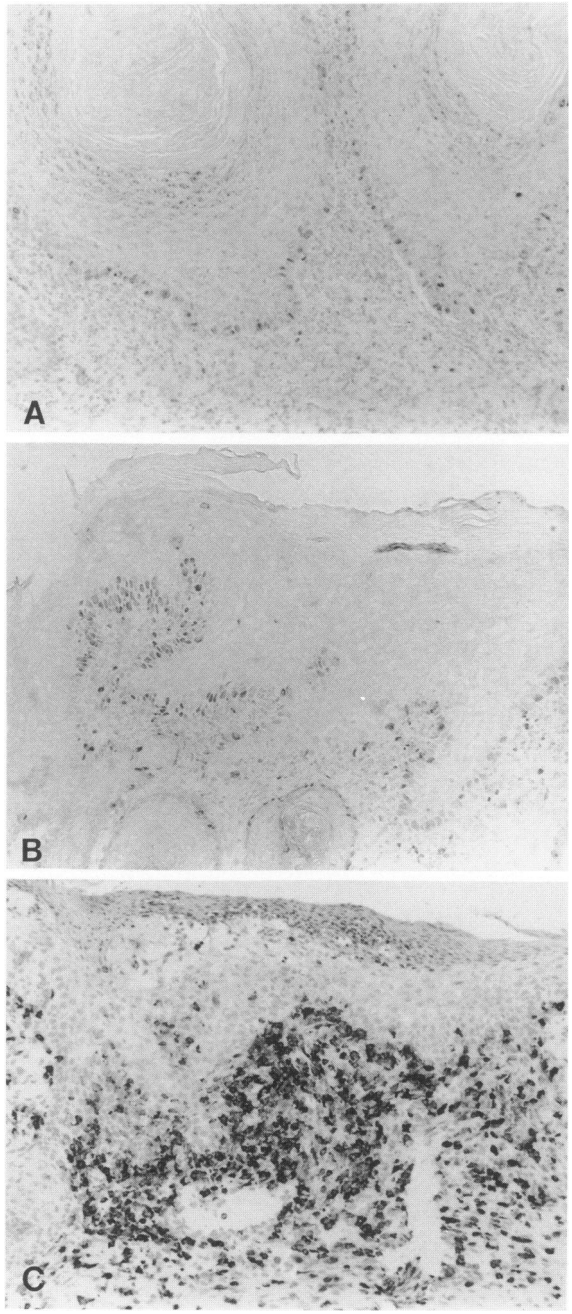

Figure 3. *Distribution of proliferating cells in regressing papillomas.* **A, B, C:** *MAb-019 (anti-BrdU), Ki-67, and L11/135 (T cell), respectively. In regressing papillomas, most of the MAb-019* **(A)**- *and Ki-67* **(B)**-*positive papilloma cells can be seen near the basement membrane in the epidermis (mainly in rank 3), whereas there are a few positive cells in the upper epidermis. Infiltrating leukocytes can be seen in both the dermis and epidermis of the section. Few of the dermal leukocytes are MAb-019- and Ki-67-positive, whereas the epidermal leukocytes exhibited a higher percentage of positive cells. Epidermal leukocytes present consist mostly of L11/135-positive T cells* **(C)**. *Magnification, ×130.*

3 (Table 1, Figure 2, A and B). The upper epidermis in each section (rank 1 or rank 2) had a higher percentage of positive cells than the basal layer (rank 3), but no significant differences could be seen. Each rank in all progressing papillomas contained a greater number of Ki-67- than MAb-019-positive cells ($P < 0.01$).

In regressing papillomas, these two antibodies showed remarkably different labeling distributions as compared with progressing papillomas (Table 1). In rank 3, there was no significant difference between progressing and regressing papillomas with respect to numbers of MAb-019- and Ki-67-positive cells. However, in ranks 1 and 2, regressing papillomas had an extremely decreased number of MAb-019- and Ki-67-positive cells (6.6% and 7.9% in rank 2, 3.8% and 2.9% in rank 1, respectively), as compared with ranks 1 and 2 in progressing papillomas, which contained a slightly higher percentage of positive cells than did rank 3. There were also significant differences in MAb-019- and Ki-67-positive cells between rank 1 or rank 2 and rank 3 ($P < 0.01$).

Immunoreactivity with MAb-019 and Ki-67 was examined in all tissue sections taken from Shope papillomas and various normal organs as positive controls. Immunohistochemical staining was predominantly nuclear in MAb-019- and Ki-67-positive cells, whereas cytoplasmic staining was also observed in epidermal basal layers by the Ki-67 MAb. Only positive nuclei were counted. These two antibodies showed very similar tissue distributions. For example, in brain, cerebellum, liver, and kidney, immunoreactivity to both MAb-019 and Ki-67 was not seen at all. On the other hand, in stomach and colon, squamous epithelial cells did not exhibit positive staining, whereas glandular cells reacted with both MAb-019 and Ki-67 MAbs. Although there was no significant difference in the staining patterns in normal tissues and in regressing papillomas, it appeared that the Ki-67 MAb labeled a slightly higher number of cells than MAb-019. There was a significant difference, however, in the number of positive cells in progressing papillomas between MAb-019 and Ki-67 ($P < 0.01$).

Analysis of Proliferation of Dermal and Epidermal Leukocytes in Progressing and Regressing Papillomas

As we have previously reported,[8] the dermis of progressing papillomas contains few B and T cells. These dermal leukocytes in progressing papillomas (ranks 4, 5, and 6) did not react with MAb-019 and Ki-67 MAbs; the percentage of positive cells ranged only from 0 to 0.5%, respectively. It was difficult to distinguish infiltrating leukocytes in the epidermis from papilloma cells by manual immunostaining. To allow for better resolution of stained sections, a computer-driven Code-On Immunostainer (Instrumentation Laboratory, Lexington, MA) was successfully

Table 1. *Comparison of Tumor Cell Proliferation Between Regressing and Progressing Papillomas*

Rank	MAb-019 (BrdU)		Ki-67	
	Regressor (n = 4)*	Progressor (n = 17)	Regressor (n = 3)	Progressor (n = 15)
1 (superficial epidermis)	1.7 ± 2.4/44.5 ± 9.2† (3.8%)	15.1 ± 4.1/51.2 ± 10.4 (20.9%)‡	1.3 ± 1.6/45.2 ± 1.2 (2.9%)	26.6 ± 7.2/38.9 ± 10.6 (68.4%)‡
2 (mid-epidermis)	4.0 ± 3.8/61.0 ± 9.0 (6.6%)	22.5 ± 5.0/72.1 ± 13.6 (31.2%)‡	4.2 ± 4.2/53.1 ± 1.8 (7.9%)	30.7 ± 7.6/53.2 ± 12.8 (57.5%)‡
3 (deep epidermis)	17.9 ± 1.4/59.4 ± 4.7 (30.1%)	17.5 ± 4.2/61.2 ± 6.5 (28.6%)	45.1 ± 16.3/66.3 ± 16.1 (68.0%)	26.7 ± 8.0/54.5 ± 7.7 (49.0%)

* Number of papillomas examined from 3 regressor rabbits and 6 progressor rabbits.
† Number of positive cells ± SD/mean number of total tumor cells ± SD in each rank. Numbers in parentheses, percentage of positive cells to total cells.
‡ $P < 0.05$ Student's t-test; the percentage of each MAb-positive cells was compared between regressing and progressing papillomas of each rank.

used. Intraepidermal lymphocytes had small (one-half to one-third of that of tumor cells), round, homogenous, dense, blue-colored nuclei by hematoxylin counterstaining, whereas the nuclei of papilloma cells were different. Usually few leukocytes were seen in the epidermis of progressing papillomas (Figure 2C). Therefore, positive cells could not be counted by rank, but instead examined as a function of the total number of epidermal leukocytes in ranks 1 to 3 combined.

The percentage of leukocytes in the epidermis of progressing papillomas labeled by MAb-019 was 4.5 ± 1.1% (mean ± SD), and Ki-67-positive cells were 6.8 ± 1.8% (mean ± SD). Although these two values were relatively low, significant differences could be seen between MAb-019- and Ki-67-positive cells in the epidermis and in the dermis (ranks 4, 5, and 6) (Table 2). About 20% of the leukocytes in the dermis (ranks 4, 5, and 6) were T cells, which were stained by L11/135 MAb. In contrast, 93.3% of intraepidermal lymphocytes consisted of L11/135 MAb-positive T cells. This result indicates that almost all infiltrating leukocytes in the epidermis were T cells, and 4.2 to 6.3% of those were proliferating. On the other hand, regressing papillomas contained many infiltrating leukocytes in the dermis and epidermis. More than 70% of the dermal leukocytes (in ranks 4, 5, and 6) consisted of T cells labeled by the L11/135 MAb (Table 2, Figure 3C), and almost the same percentage of those cells also expressed class II antigen, as we have previously reported.[8] As shown in Table 2, only a small percentage of the dermal leukocytes in regressing papillomas were labeled by MAb-019 (0.6% in rank 4 to 1.2% in rank 6) and Ki-67 (1.5% in rank 5 to 2.7% in rank 6). Most of the cells did not react with either of the two MAbs, despite expressing

Table 2. *Quantitation of Proliferating Leukocytes in the Epidermis and the Dermis of Progressing and Regressing Papillomas*

Rank	MAb-019 (BrdU)		Ki-67		L11/135 (T cells)	
	Progressing papillomas (n = 17)*	Regressing papillomas (n = 4)	Progressing papillomas (n = 15)	Regressing papillomas (n = 3)	Progressing papillomas (n = 16)	Regressing papillomas (n = 3)
1 (superficial epidermis)		0.2 ± 0.2/ 2.3 ± 0.4† (8.7%)§		0.6 ± 0.3/ 4.5 ± 0.7 (13.3%)		5.9 ± 2.2/ 6.4 ± 2.2 (92.2%)
2 (mid-epidermis)	Combined ranks 1–3 (4.5%)‡§	0.7 ± 0.2/ 9.0 ± 2.6 (7.8%)§	Combined ranks 1–3 (6.8%)§	4.0 ± 2.4/ 20.3 ± 5.8 (19.7%)	Combined ranks 1–3 (93.3%)	18.1 ± 3.6/ 19.1 ± 3.7 (94.8%)
3 (deep epidermis)		1.9 ± 0.5/ 16.7 ± 2.7 (11.4%)§		8.1 ± 1.5/ 28.8 ± 5.7 (28.1%)§		23.7 ± 5.2/ 24.1 ± 5.2 (98.3%)
4 (superficial dermis)	0.0 ± 0.1/ 26.9 ± 3.5 (0%)	0.3 ± 0.3/ 54.2 ± 5.7 (0.6%)	0.0 ± 0.1/ 24.7 ± 2.9 (0%)	1.1 ± 0.1/ 64.2 ± 2.7 (1.7%)	4.8 ± 1.4/ 23.4 ± 2.8 (20.5%)	53.6 ± 8.0/ 69.7 ± 7.6 (76.9%)
5 (mid-dermis)	0.2 ± 0.1/ 43.8 ± 5.0 (0.5%)	0.5 ± 0.2/ 75.7 ± 7.5 (0.7%)	0.1 ± 0.1/ 40.9 ± 5.0 (0.2%)	1.3 ± 0.4/ 29.2 ± 3.2 (1.5%)	7.0 ± 2.0/ 38.8 ± 4.6 (18.0%)	72.7 ± 10.9/ 99.1 ± 12.6 (73.4%)
6 (deep dermis)	0.1 ± 0.1/ 41.1 ± 5.6 (0.2%)	0.6 ± 0.6/ 59.8 ± 5.6 (1.2%)	0.1 ± 0.1/ 39.9 ± 5.2 (0.3%)	1.9 ± 0.4/ 69.9 ± 4.9 (2.7%)	6.9 ± 1.8/ 36.3 ± 5.6 (19.0%)	55.6 ± 10.8/ 79.5 ± 8.7 (69.9%)

* Number of progressing and regressing papillomas examined from 6 progressor and 3 regressor rabbits, respectively.
† Number of positive cells ± SD/mean number of total leukocytes ± SD in each rank.
‡ Percentage of positive leukocytes to total epidermal leukocytes. Numbers in parentheses, percentage of positive leukocytes to total leukocytes per rank.
§ $P < 0.05$; P value of the percentage of MAb-019- and Ki-67-positive cells when compared between the epidermis (ranks 1–3) and the dermis (ranks 4–6).

Table 3. *Summary of the Changes in Regressing Papillomas as Compared with Progressing Papillomas*

Proliferating tumor cells in epidermis	Number of T cells in epidermis	Proliferating T cells in epidermis	Number of T cells in dermis	Proliferating T cells in dermis
Decreased	Increased	Increased	Increased	Decreased

class II antigen. On the other hand, epidermal infiltrating leukocytes had a higher number of MAb-019-positive cells (7.8% in rank 2 to 11.4% in rank 3) and Ki-67-positive cells (15.2% in rank 1 to 28.1% in rank 3). Most of the infiltrating cells in the epidermis were located near the basement membrane (rank 3).

When the immunoreactivity of MAb-019 and Ki-67 was compared with infiltrating leukocytes, leukocytes in the epidermis (ranks 1, 2, and 3) contained a higher percentage of positive cells than those in the dermis. There was a significant difference in immunoreactivity with MAb-019 and Ki-67 between the epidermis (except rank 1 of Ki-67) and the dermis ($P < 0.01$). Epidermal infiltrating leukocytes consisted mainly of T cells (92.2% in rank 1 to 98.3% in rank 3) (Table 2, Figure 3C), whereas almost no B cells could be seen (data not shown). The percentage of T cells (relative to the total number of infiltrating leukocytes) in the epidermis of regressor papillomas was similar to that of progressor papillomas. These results are summarized in Table 3.

Discussion

We hypothesized that cellular proliferation in regressing papillomas was suppressed or reduced relative to that in progressing lesions. Our previous study suggested that tumor cell growth might be suppressed in regressing papillomas when compared with progressing papillomas as determined by counting tritiated thymidine-labeled tumor cells.[7] In the current study, these findings were independently confirmed as demonstrated by the immunohistochemical reactivity pattern seen with MAb-019 and Ki-67 MAbs.[7] Our data demonstrated that cellular proliferation occurred only near the basement membrane in the epidermis of regressing papillomas (eg, germinal layer), and that this proliferation was suppressed in the upper layers of regressing papillomas (eg, stratum spinosum or stratum granulosum). The suppression of cellular proliferation in the stratum spinosum and granulosum but not in the germinal layer raises an interesting question. Dense leukocytic infiltrates are concentrated mainly near the basement membrane in the dermis or in the epidermis. If these infiltrating lymphocytes and the keratinocytes near the basement membrane produced cytostatic/cytotoxic lymphokines such as interferon and tumor necrosis factor-α, which are toxic to papillomas, it might be assumed that tumor suppression would be greater in the lower epidermal layers of the papilloma. The question is then why tumor growth is suppressed in the upper layers and not in the lower layers.

The answer to this question might offer insight into the mechanism of papilloma regression. One possible reason is that, because passive diffusion of nutrients and oxygen decreases as the distance from the dermal blood supply increases, the cells within the superficial layers of the papilloma are more susceptible to the toxic activity of such cytokines. Another possibility is that these factors may specifically affect CRPV gene expression. If cell proliferation in the superficial layers of the papilloma is driven by the expression of CRPV genes E6 and E7, which are implicated in cellular transformation, a decrease in their expression may remove infected cells from the cell cycle.

However, the method of elimination of papilloma cells in regressing lesions also remains to be defined. In this study, regions of necrosis were not observed in regressing lesions. Likewise, cell necrosis has not been observed in the examination of numerous regressing lesions by one of us (JWK). One possible mechanism for the elimination of infected cells would be through normal epidermal migration and desquamation. In this mechanism, cellular proliferation may be halted and/or differentiation initiated by the release of leukocytic factors as mentioned above. Early studies by Rashad and Evans[21] have indicated that cell migration rates within Shope papillomas may be greater than that seen for normal rabbit epidermal basal cells. If one thinks of papilloma growth as an imbalance of epidermal proliferation versus epidermal migration/desquamation, then by decreasing only the proliferative fraction of the lesion the balance would be shifted so that the rate of cell loss would exceed the rate of cell replacement and the lesion would then slowly disappear. This model does not require the death of infected cells to account for gradual lesion reduction, but we cannot now exclude a role for cytoxic T cells.

Monoclonal antibodies to BrdU–DNA complexes (MAb-019) and the MAb Ki-67 are useful alternatives to isotopic methods to detect proliferating cells.[22–25] In this study, we compared the staining patterns of these antibodies in normal organs and in both progressing and regressing Shope papillomas induced

by CRPV. Although these two antibodies exhibited similar staining patterns and distribution in each tissue, the Ki-67 MAb always labeled a higher percentage of cells than MAb-019. This difference was remarkable in progressing papillomas ($P < 0.01$). This result may reflect the different phases of the cell cycle sensitive to MAb-019 or Ki-67 labeling. Ki-67 antigen is produced during all cell cycle phases except G0,[15] but incorporation of BrdU into DNA is mainly restricted to S-phase cells.[11] In turn, the Ki-67 MAb might be more sensitive than MAb-019 in the detection of actively proliferating cells (ie, tumors), because of the lower background staining exhibited.

In some other diseases, for example psoriasis,[26] delayed-type hypersensitivity,[27] and inflammatory dermatoses,[28] infiltration of T lymphocytes has also been observed in the lesions. In addition, in mycosis fungoides and cutaneous T cell lymphoma, a dense infiltration of T cells was reported.[24,29–31] These T cells express lymphocyte function-associated antigen (LFA-1), which is a member of the integrin receptor family of molecules,[35,36] on their cell surfaces.[32–34] LFA-1 present on T cells interacts with the intercellular adhesion molecule-1 (ICAM-1) on the surface of keratinocytes after trafficking through the high endothelial cells lining the post-capillary venules of the skin.[26,34] However, it was reported that in mycosis fungoides, there was discordant phenotype expression of T cells and Ki-67 antigen expression between intraepidermal and intradermal T cells.[24,31] We attempted to stain regressing rabbit lesions with MAbs that recognize human LFA-1, ICAM-1, and Ki-67, but only the MAb Ki-67 cross-reacted with rabbit cells. Immunostaining with MAb-019 and Ki-67 showed that there were few proliferating T cells in the dermis, despite expression of class II antigen, whereas the epidermal T cells nuclei reacted with the Ki-67 antigen. Intratumoral T cells were easily distinguished from papilloma cells on hematoxylin and eosin-stained sections by the small, round, homogenous, and dense blue staining characteristics of their nuclei and by immunostaining with MAb-019. More than 90% of the cells that had these nuclear characteristics were stained with the rabbit T cell-specific L11/135 antibody (Table 3). We did not observe necrotic foci, which might have made interpretation of the cell counts more difficult. Although we could not demonstrate the existence of LFA-1 on the dermal T cells, this glycoprotein might be expressed in order for T cells to enter the epidermis after the interaction with the ICAM-1 on the surface of the keratinocytes. However, additional studies are needed to determine whether epidermal proliferating T cells have a different function for lymphokine production from the dermal T cells.

Our data conclusively demonstrate that in regressing papillomas tumor cell proliferation was strongly suppressed in the upper layers of the epidermis. This mechanism might be induced by the production of lymphokines from infiltrating T cells (especially intraepidermal T cells). In general, human flat warts or cervical neoplasms caused by human papillomavirus frequently undergo spontaneous regression. The examination of Shope papilloma regression is a useful model for understanding spontaneous human papilloma regression and the role of host immunity.

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References

1. zur Hausen H: Human papillomaviruses and their possible role in squamous cell carcinomas. Curr Top Microbiol Immunol 1977, 78:1–30
2. Kreider JW, Howett MK, Wolfe SA, Bartlett GL, Zaino RJ, Sedlacek TV, Mortel R: Morphological transformation *in vivo* of human uterine cervix with papillomavirus from condylomata acuminata. Nature 1985, 317:639–641
3. Yasumoto S, Burkhardt AL, Doniger J, DiPaolo JA: Human papillomavirus type 16 DNA-induced malignant transformation of NIH 3T3 cells. J Virol 1986, 57:572–577
4. Kreider JW: Studies on the mechanism responsible for the spontaneous regression of the Shope rabbit papilloma. Cancer Res 1963, 23:1593–1599
5. Evans CA, Ito Y: Antitumor immunity in the Shope papilloma-carcinoma complex of rabbits. III. Response to reinfection with viral nucleic acid. JNCI 1966, 36:1161–1166
6. Tagami H, Ogino A, Takigawa M, Imamura S, Ofuji S: Regression of plane warts following spontaneous inflammation—a histopathological study. Br J Dermatol 1974, 90:147–154
7. Kreider JW: Neoplastic progression of the Shope rabbit papilloma. Viruses in Naturally Occurring Cancers (A). Edited by M Essex, G Todaro, and H zur Hausen. Cold Spring Harbor Conference on Cell Proliferation, vol 7. Cold Spring Harbor, Cold Spring Harbor Press, 1980
8. Okabayashi M, Angell MG, Christensen ND, Kreider JW: Morphometric analysis and identification of infiltrating leukocytes in regressing and progressing Shope rabbit papillomas. Int J Cancer 1991, 49:919–923
9. Kember NF: Cell division in endochondral ossification—a study of cell proliferation in rat bones by the method of tritiated thymidine autoradiography. J Bone Joint Surg [B] 1960, 42:824–839

10. Pierard GE, Pierard-Franchimont C: The proliferative activity of cells of malignant melanomas. Am J Dermatopathol 1984, 6:317–323
11. Gratzner HG: Monoclonal antibody to 5-bromo- and 5-iododeoxy-uridine: a new reagent for detection of DNA replication. Science 1982, 218:474–475
12. Morstyn G, Hsu SM, Kinsella T, Gratzner H, Russo A, Mitchell JB: Bromodeoxyuridine in tumors and chromosomes detected with a monoclonal antibody. J Clin Invest 1983, 72:1844–1850
13. deFazio A, Tattersall MHN: Rapid fluorometric detection of drug resistant tumour cells. Br J Cancer 1985, 52:633–636
14. Gerdes J, Schwab U, Lemke H, Stein H: Production of a mouse monoclonal antibody reactive with a human nuclear antigen associated with cell proliferation. Int J Cancer 1983, 31:13–20
15. Gerdes J, Lemke H, Baisch H, Wacker H-H, Schwab U, Stein H: Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. J Immunol 1984, 133:1710–1715
16. Weiss LM, Strickler JG, Medeiros LJ, Gerdes J, Stein H, Warnke RA: Proliferative rates of non-Hodgkin's lymphomas as assessed by Ki-67 antibody. Hum Pathol 1987, 18:1155–1159
17. Brown DC, Gatter KC: Monoclonal antibody Ki-67: its use in histopathology. Histopathology 1990, 17:489–503
18. Gerdes J, Li L, Schlueter C, Duchrow M, Wohlenberg C, Gerlach C, Stahmer I, Kloth S, Brandt E, Flad H-D: Immunobiochemical and molecular biologic characterization of the cell proliferation-associated nuclear antigen that is defined by monoclonal antibody Ki-67. Am J Pathol 1991, 138:867–873
19. Apte SS: Ki-67 monoclonal antibody (MAb) reacts with a proliferation associated nuclear antigen in the rabbit *Oryctolagus cuniculus*. Histochemistry 1990, 94:201–204
20. Jackson S, Chused TM, Wilkinson JM, Leiserson WM, Kindt TJ: Differentiation antigens identify subpopulations of rabbit T and B lymphocytes: definition by flow cytometry. J Exp Med 1983, 157:34–46
21. Rashad AL, Evans CA: Radioautographic study of epidermal cell proliferation and migration in normal and neoplastic tissues of rabbits. JNCI 1968, 41:845–853
22. Veronese S, Gambacorta M, Falini B: *In situ* demonstration of tissue proliferative activity using anti-bromodeoxyuridine monoclonal antibody. J Clin Pathol 1989, 42:820–826
23. McDowell EM, Zhang XM, Desanti AM: Localization of healing tracheal wounds using bromodeoxyuridine immunohistochemistry. Stain Technol 1990, 65:25–29
24. Nickoloff BJ, Griffiths CEM: Intraepidermal but not dermal T lymphocytes are positive for a cell-cycle-associated antigen (Ki-67) in mycosis fungoides. Am J Pathol 1990, 136:261–266
25. Porschen R, Kriegel A, Langen C, Classen S, Hilse M, Lohe B, Hengels KJ, Borchard F: Assessment of proliferative activity in carcinomas of the human alimentary tract by Ki-67 immunostaining. Int J Cancer 1991, 47:686–691
26. Chin YH, Falanga V, Cai JP: Lymphocyte adhesion to psoriatic dermal endothelium: mechanism and modulation. J Invest Dermatol 1990, 95:29S-31S
27. Poulter LW, Seymour GJ, Duke O, Janossy G, Panayi G: Immunohistological analysis of delayed-type hypersensitivity in man. Cell Immunol 1982, 74:358–369
28. Markey AC, Allen MH, Pitzalis C, MacDonald DM: T-cell inducer populations in cutaneous inflammation: a predominance of T helper-inducer lymphocytes (THi) in the infiltrate of inflammatory dermatoses. Br J Dermatol 1990, 122:325–332
29. Lutzner M, Edelson R, Schein P, Green I, Kirkpatrick C, Ahmed A: Cutaneous T-cell lymphomas: the Sezary syndrome, mycosis fungoides, and related disorders. Ann Intern Med 1975, 83:534–552
30. Miller RA, Coleman CN, Fawcett HD, Hoppe RT, McDougall IR: Sezary syndrome: a model for migration of T lymphocytes to skin. N Engl J Med 1980, 303:89–92
31. Michie SA, Abel EA, Hoppe RT, Warnke RA, Wood GS: Discordant expression of antigens between intraepidermal and intradermal T cells in mycosis fungoides. Am J Pathol 1990, 137:1447–1451
32. Shiohara T, Moriya N, Saizawa K, Gotah C, Yagita H, Nagashima M: Evidence for involvement of lymphocyte function-associated antigen 1 in T cell migration to epidermis. J Immunol 1991, 146:840–845
33. Nickoloff BJ, Mitra RS: Phorbol ester treatment enhances binding of mononuclear leukocytes to autologous and allogeneic γ-interferon-treated keratinocytes, which are blocked by anti-LFA-1 monoclonal antibody. J Invest Dermatol 1988, 90:684–689
34. Singer KH: Interactions between epithelial cells and T lymphocytes: role of adhesion molecules. J Leukocyte Biol 1990, 48:367–374
35. Hynes RO: Integrins: a family of cell surface receptors. Cell 1987, 48:549–554
36. Figdor CG, van Kooyk Y, Keizer GD: On the mode of action of LFA-1. Immunol Today 1990, 11:277–280