Re-expression of Interleukin 1 in Human Papillomavirus 18 Immortalized Keratinocytes Inhibits Their Tumorigenicity in Nude Mice

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Abstract
The ability to form tumors in nude mice developed spontaneously in the human papillomavirus (HPV)-18 immortalized keratinocyte cell line, 18-11, and is shown here to be accompanied by a loss of interleukin 1 (IL-1) α and β expression at both the RNA and protein level. In addition, a separate tumorigenic 18-11 derivative and two cervical carcinoma-derived cell lines, HeLa and Caski, were found to have significantly decreased or lost IL-1α and IL-1β expression. Using retroviral expression vectors, we re-established IL-1 expression in tumorigenic 18-11 cells (18-11S3) in an effort to evaluate whether loss of IL-1 expression represented an important phenotypic change in the development of tumorigenicity in these cells. IL-1-expressing 18-11S3 cells showed a range of tumorigenic potential, depending on the type and combination of IL-1α and IL-1β expressed. Although 18-11S3 expressing the precursor forms of both IL-1α and IL-1β normally found in keratinocytes showed moderate inhibition of tumorigenicity, other IL-1-expressing lines showed complete inhibition of tumor formation. Co-injection of nontumorigenic, IL-1-expressing 18-11S3 with parental 18-11S3 also inhibited tumor formation. These results suggest that maintenance of IL-1 expression may play an important role in preventing progression to tumorigenicity in cervical carcinoma and other epithelial cancers.

Introduction
High-risk types of HPV are associated with nearly all cervical carcinomas (1, 2). Although expression of the E6 and E7 open reading frames from high-risk HPV types can immortalize epithelial cells, it has become apparent that additional cellular alterations must take place before these cells can progress to tumorigenicity (3–5). The 18-11 cell line is a foreskin keratinocyte cell line that was immortalized by expression of the E6 and E7 proteins from HPV 18 (6, 7). A tumorigenic derivative of this cell line (18-11S3) developed spontaneously after long-term passage in tissue culture (4). These cells form progressive and invasive tumors when injected s.c. in nude mice. Herein, we show that expression of the multifunctional cytokine proteins IL-1α and IL-1β are lost concomitant with the development of tumorigenicity in the 18-11 cell line.

IL-1 is a pleiotropic factor that exists in two distinct but functionally related forms: IL-1α and IL-1β. Both of these proteins are produced in a precursor form that is proteolytically cleaved to produce bioactive mature proteins (precursor IL-1α (pro-IL-1α) possesses biological activity, while it is generally believed that precursor IL-1β (pro-IL-1β[proβ]) does not) that interact with a common receptor (8). The role of IL-1 production in epithelium is incompletely understood, although a number of recent studies are beginning to shed light on this subject. It has been suggested that IL-1 production by the Langerhans cells of epidermis may play an important role as a costimulatory signal in the activation of T-cell responses in epithelium (9). This costimulatory effect of IL-1 is well characterized for other antigen-presenting cells such as macrophages (10–13). Furthermore, exogenously added or fibroblast-derived IL-1 has been shown to activate cytolytic activity in natural killer and lymphokine-activated killer cells, which are thought to play an important role in tumor surveillance (14–16). In addition to its actions as an activator of immune effector cells, IL-1 is also reported to act as a chemottractant for lymphocytes and can effect the expression of several proteins with important functional properties, such as intercellular adhesion molecule-1, in a variety of other cell types including endothelial cells, keratinocytes, and fibroblasts (17–21). Although keratinocyte-derived IL-1 has not yet been implicated in many of these IL-1-associated activities, a few recent studies have begun to identify a role for keratinocytes in the induction of immune responses within epithelia (20, 22–26).

With these IL-1-associated activities in mind, we did additional studies aimed at determining whether re-establishment of IL-1 expression in the tumorigenic 18-11S3 cell line would lead to a concomitant reduction in its tumorigenicity. Indeed, IL-1 production by transformed fibroblasts has been reported previously to inhibit their tumorigenicity, whether produced endogenously or via gene transfer (14, 27, 28). We have used retroviral vectors to re-establish expression of both IL-1α and IL-1β either alone or in combination in 18-11S3 cells. Although expression of both precursor forms of IL-1α and IL-1β (which are reported to be the normally expressed IL-1 proteins in keratinocytes) only led to a moderate
inhibition of tumorigenicity by IL-1

Reduction in tumorigenicity of 18-11S3 cells in nude mice, other IL-1 expression patterns completely inhibited the tumorigenicity of these cells. Our results show that even in the absence of T cell-mediated antitumor activities, keratinocyte-derived IL-1 expression can inhibit tumor formation, suggesting that IL-1 expression may play an important role in preventing the development of tumors in the cervix and epithelia in general.

Results

Northern Analysis of IL-1 Expression. RNA from nontumorigenic and tumorigenic 18-11 cells were analyzed for IL-1α and IL-1β expression. The nontumorigenic 18-11S1 and 18-11S2 cells showed abundant IL-1α and IL-1β transcripts, whereas no IL-1α or IL-1β RNA could be detected in tumorigenic 18-11S3 cells (Fig. 1). These results demonstrate that loss of IL-1 expression correlates with development of tumorigenicity in the 18-11 cell line. As can be seen in Fig. 1, IL-1 RNA levels are similar when 18-11S1 and S2 cells are compared to normal foreskin keratinocytes (HFE; Fig. 1). This figure also shows that an 18-11S1 cell line that was converted to tumorigenicity by treatment with the carcinogen NMU (18-11NMU-T2) also lacks IL-1α and IL-1β expression (Fig. 1). IL-1α and IL-1β transcripts were also absent in the HPV-positive, tumor-derived HeLa and Caski cell lines.

Fig. 1. Northern analysis of IL-1α and IL-1β expression. Ten μg of total RNA were loaded in each lane. Autoradiographs were exposed for 5–14 days. In A, tumorigenic passages of the 18-11 cell line and the Caski and HeLa cell lines are negative for IL-1 expression. In B, IL-1 expression in normal keratinocytes (HFE) and 18-11 cells cultured in normal medium or media that had been conditioned for 24 h in culture with 18-11S3 cells (CM) are shown. 18-11NMU-T2 cells were derived from a tumor produced by NMU-treated 18-11S1 cells. *, tumorigenic 18-11 cell lines.

Taken together, these results suggest the possibility that loss of IL-1 expression may be a common characteristic in HPV-associated tumors. RNA from HFE, 18-11S1, and 18-11S2 cells that had been cultured for 24 h in conditioned medium from 18-11S3 cultures was also analyzed to test for the possible presence of a factor produced by 18-11S3 that inhibited IL-1 expression. Neither IL-1α or IL-1β expression appeared to be inhibited by this treatment, however, suggesting that no such factor is produced by 18-11S3 (Fig. 1, CM samples). We also performed a Southern analysis of the IL-1α and IL-1β genes to determine whether alteration in these genes might be responsible for loss of IL-1 expression in the 18-11S3 cell line. Restriction patterns for IL-1β revealed no differences between HFEs, 18-11S1, and 18-11S3, as was also the case with IL-1α in 18-11S1 and S3 cells (data not shown). In comparing IL-1α restriction patterns, two extra fragments appeared in 18-11S1 and S3 compared to HFEs, suggesting that there may have been a polymorphism in the keratinocytes used to produce the 18-11 cell line. Nevertheless, our data suggest that some cell-associated activity inhibits the transcription of IL-1α and IL-1β in the tumorigenic 18-11S3. It should be noted, however, that it is also possible that the half-life of IL-1 RNA is greatly reduced in these cells. Control of expression of IL-1 and other related cytokines at the posttranscriptional level has been reported previously (29, 30).
**18-11 IL-1 Protein Expression.** Loss of IL-1α and IL-1β protein production in 18-11S3 was confirmed by ELISA analysis of cytoplasmic proteins (Fig. 2A). Furthermore, these data demonstrate that IL-1α and IL-1β protein production in 18-11S1 cells is essentially identical to that seen in normal foreskin keratinocytes (Fig. 2A). The bioactivity of the IL-1 produced by these cell lines was also evaluated. Bioassays showed that 18-11S1s produced as much IL-1-associated activity as normal keratinocytes (Fig. 2C). As expected, 18-11S3 cells possessed no IL-1 bioactivity. These assays did not distinguish between bioactivity produced by IL-1α and IL-1β.

**Establishing IL-1 Expression in 18-11S3.** We hypothesized that loss of IL-1 expression may represent an important and potentially necessary step in the development of tumorigenicity in the 18-11S3 cell line. To test this hypothesis, we
re-established IL-1 expression in 18-11S3 so that we could evaluate the tumorigenicity of such cells in nude mice. Mature and pro-IL-1α (αM or αF, respectively) and mature and pro-IL-1β (βM or βF, respectively) encoding retroviruses were used to infect subconfluent 18-11S3 cells. These cells were selected, colonies were picked and expanded, and IL-1 production was assessed by ELISA analysis of cytoplasmic proteins (Fig. 2). Clones that expressed IL-1 at levels comparable to that seen in 18-11S1 and normal keratinocytes were chosen and either tested for tumorigenicity or superinfected with another IL-1-encoding retrovirus (which encoded a unique selectable marker). These cells were selected, and cytoplasmic proteins were again evaluated by ELISA for production of both IL-1α and IL-1β species present in the final clone. For each of the different IL-1 species, either alone or in combination, clones were isolated which produced IL-1 at or near levels seen in 18-11S1 and normal keratinocytes (Fig. 2A). In addition, several clones were produced that were sequentially infected with retroviruses containing vectors that did not encode IL-1 but did encode hygromycin, neomycin, or puromycin resistance. These cell lines produced minimal or no IL-1; one such cell line is shown (HP; Fig. 2).

Production of extracellular IL-1 by these cell lines was also assessed. The αFβM cell line produced significant extracellular IL-1β (Fig. 2B). In Fig. 2C, an analysis of cytoplasmic proteins shows that several of the IL-1-producing 18-11S3 cell lines produce some degree of IL-1 bioactivity. Very limited or no IL-1 activity is detected in parental 18-11S3 or vector-infected HP cells (Fig. 2C).

**IL-1 Expression Reduces Tumorigenicity of 18-11S3.** Parental 18-11S3, vector-infected positive controls, and IL-1-expressing 18-11S3 cells were tested for tumorigenicity in nude mice. Parental 18-11S3 and vector-infected cells (HP; Fig. 3) produced progressively growing tumors in all mice (Fig. 3). Four other vector-infected cell lines were also tested in groups of three to five mice, and tumor growth was observed in all of these mice (data not shown). In one group, however, one of five mice showed complete regression of its
Fig. 4. A, tumorigenicity of mixed HP and 18-11S3 cells (HP-CI) and of mixed αFBM and 18-11S3 cells (αFBM-CI). B, tumorigenicity of 18-11S3 cells when injected on the opposite side of nude mice that had been pre-injected 2 weeks earlier with either HP cells (HP-PI) or αFBM cells (αFBM-PI). Tumor growth shown is at the site of 18-11S3 cell injection.

Summary of Tumor Mixing Data

<table>
<thead>
<tr>
<th>Cell Line</th>
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<tr>
<td>HP-CI</td>
<td>2/2</td>
<td>HP-PI</td>
<td>2/2</td>
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<tr>
<td>αFBM-CI</td>
<td>1/3</td>
<td>αFBM-PI</td>
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Tumor at 10 weeks after injection, suggesting that occasional clones from parental 18-11S3 may exhibit slightly reduced tumorigenicity (i.e., ~80% tumor formation). The tumorigenicity of IL-1-expressing 18-11S3 could be assigned to roughly three groups. 18-11S3 expressing pro-IL-1α (αF) retained full tumorigenic potential (Fig. 3). On the other hand, cells expressing pro-IL-1α and mature IL-1β (αFBM) showed complete inhibition of tumorigenicity, whereas cells expressing pro-IL-1β (βF) alone or both precursor forms of IL-1α and IL-1β (αFBFM) exhibited an intermediate inhibition of tumorigenicity (Fig. 3). A cell line expressing mature IL-1α and pro-IL-1β also showed complete inhibition of tumorigenicity (data not shown). Thus, it would appear that IL-1α expression by itself does not inhibit tumorigenicity in these cells, whereas IL-1β expression on its own can inhibit tumorigenicity to some degree. When both IL-1α and IL-1β are expressed in certain combinations, complete inhibition of tumorigenicity can be produced. In addition, it appears that the level of IL-1 production may be important because a second αFBM cell line, which produced only 20–30% of the IL-1 produced by the αFBM cell line shown in Fig. 3, did form tumors in three of six mice (data not shown). It should also be noted that tumor formation can apparently occur in the presence of IL-1 production because cells cultured from a number of tumors showed ELISA levels of IL-1 similar to that seen in the cells prior to injection (data not shown). Nevertheless, these results demonstrate that expression of IL-1 in a variety of combinations in 18-11S3 can significantly inhibit the tumorigenicity of this cell line.

Mixed Tumor Transplantation Assay Demonstrates Local Effect of IL-1-producing Cells. To determine whether IL-1 production by the cells themselves was necessary to inhibit tumor formation, we tested the ability of parental 18-11S3 cells to form tumors either when injected in a 1:1 mixture with vector-infected HP or non-tumorigenic αFBM cells or when injected into the opposite shoulder of a mouse that had received an equal amount of HP or αFBM cells 2 weeks earlier. Progressively growing tumors developed at the injection site of 18-11S3 cells (in mixture or on opposite shoulder) in all mice that also received HP cells (Fig. 4). Tumors also formed in mice that received 18-11S3 cells 2 weeks after injection of αFBM cells on the opposite shoulder (Fig. 4). However, when a mixture of 18-11S3 and αFBM cells were injected, only one of three mice formed a tumor (Fig. 4).
These results suggest that IL-1-expressing cells can produce a non-cell autonomous, local antitumor effect, although no systemic effect was observed. We speculate that such non-tumorigenic IL-1-expressing cell lines may inhibit tumorigenicity by activating tumoricidal activity from macrophage and natural killer cell populations. Such a mechanism of tumor inhibition would fit with our observation of a non-cell autonomous, local antitumor effect seen in the mixed tumor transplantation assay; however, a direct demonstration of such a mechanism awaits future studies.

Discussion

We have shown that concomitant with the development of tumorigenicity in the HPV-18 immortalized keratinocyte cell line 18-11, expression of the IL-1α and IL-1β proteins are also lost. In addition, we also demonstrated loss of IL-1 expression in an early-passage 18-11S1 cell line that was converted to tumorigenicity by treatment with the carcinogen NMU and in the cervical carcinoma-derived cell lines HeLa and Caski, suggesting that loss of IL-1 expression may be a common event in the development of cervical carcinoma. A few studies have addressed cytokine production by cervical carcinoma-derived cells and have found that, although not always lost completely, IL-1 expression is reduced in comparison to cells from normal cervix (31). In the tumorigenic 18-11S3 cells, loss of IL-1 expression appears to be the result of inhibition of IL-1α and IL-1β gene transcription, although it is possible that decreased RNA message stability could also play a role. Nonetheless, these results suggest that, in addition to using gene transfer to re-establish IL-1 expression in such cells, it may be possible to induce endogenous IL-1 production by pharmacological means.

As referred to earlier, IL-1, and possibly keratinocyte-derived IL-1, can help activate immune effector cells and stimulate expression of potentially important proteins that could play a large role in inhibiting tumor formation (14–26, 32, 33). Taking these activities into consideration, we postulated that loss of IL-1 expression may represent a crucial phenotypic change in the 18-11 cell line that allowed it to develop tumorigenic potential. To test this hypothesis, we used retroviral vector-mediated gene transfer to re-establish IL-1α and/or IL-1β expression in 18-11S3 cells and then tested their tumorigenicity in nude mice. Our results showed that although re-expression of pro-IL-1α alone did not inhibit tumorigenicity, re-expression of IL-1β, to some degree on its own or more significantly in combination with IL-1α, could lead to partial or complete inhibition of tumorigenicity in the 18-11S3 cell line (Fig. 3). These results seem to be in close agreement with previous studies that have established that IL-1β can act to increase antigen presentation and stimulate immune responses in epithelia, whereas IL-1α appears to actually inhibit antigen presentation in epithelia (21–25, 34, 35). The characteristics of IL-1 production in cell lines with decreased tumorigenicity are variable. Although some extracellular IL-1 bioactivity is seen in the αFBF and αFBM cell lines, none is detected in the β cell line. Cell-associated IL-1 bioactivity is seen in all of these cell lines; however, such bioactivity is also seen in the fully tumorigenic α cell line. Thus, our results seem to suggest that IL-1 bioactivity may not be an indicator of the ability of IL-1 to inhibit tumorigenicity.

Despite success in inhibiting the tumorigenicity of 18-11S3 with a number of different IL-1-expressing cell lines, our results do not conclusively establish that loss of IL-1 expression represented a necessary step in the development of tumorigenicity in 18-11S3. Since epithelial cells are thought to express only the precursor forms of IL-1α and IL-1β, our αFBF cell line would presumably most closely represent the pre-tumorigenic phenotype of the 18-11 cell line in regard to IL-1 expression. Although this cell line exhibited a moderate degree of inhibition of tumorigenicity, it did not demonstrate complete blockage of tumor formation. This may indicate that, although IL-1 production in epithelial cells can certainly decrease tumorigenic capacity, it may not represent an impassable obstacle to the development of tumorigenicity. Perhaps loss of IL-1 expression represents one of several possible phenotypic alterations that can contribute to the development of malignant potential.

Given the lack of T cell-mediated immunity in nude mice, it is not surprising that pre-injection of HP or αFBM cells did not lead to protection in these mice against subsequent injection of 18-11S3 cells at a different site. Interestingly, injection of a mixture of αFBM and 18-11S3 cells revealed an inhibition of tumor formation as compared to mice that received a mixture of HP and 18-11S3 cells. Such an observation suggests that a locally restricted yet non-cell autonomous antitumor response is elicited by IL-1-expressing cell lines such as αFBM. It is tempting to speculate that this effect may be mediated by nonspecific effector cells such as natural killer cells, although a direct demonstration of such an activity requires further study. It is also uncertain whether our observed inhibition of tumorigenicity in IL-1-expressing cells is the result of a direct activity of IL-1, or if it is mediated by enhanced expression of other cell-associated factors such as cell adhesion molecules or other cytokines. Regardless of the mechanism involved, our results show that keratinocyte-associated IL-1 production can inhibit the tumorigenicity of the 18-11S3 epithelial cell line. This suggests that maintenance of IL-1 expression in premalignant epithelial cells may play a crucial role in preventing the subsequent development of tumorigenicity in such cells.

Materials and Methods

Normal and Immortalized Keratinocyte Cell Lines. Normal foreskin keratinocyte cell lines were isolated as described previously (36, 37). The HPV-18 immortalized cell line, 18-11, was produced by transfection of HPV-18 DNA sequences into normal primary human foreskin keratinocytes (6, 7). The presence and physical state of HPV sequences has been confirmed and characterized in previous publications (6, 7, 38). All keratinocyte cell lines were grown in keratinocyte-SFM (Life Technologies, Inc., Grand Island, NY). The medium was changed every 2 days, and cells were split at a ratio of 1:3 when confluence was reached. 18-11S1 refers to 18-11 cells of passage levels 0–30, 18-11S2 refers to passage levels 30–60, and 18-11S3 refers to tumorigenic cells of passage 60 or greater. The 18-11NMU-T2 cell line is derived from a tumor formed in nude mice by 18-11S1 cells that were converted to tumorigenicity by treatment with NMU (39). Mouse fibroblast cell lines PE501 and PG13 were grown and maintained in DMEM + 5% fetal bovine serum and split at 1:10 when confluent.

IL-1α and IL-1β Retroviral Expression Vectors. cDNAs for both precursor and mature forms of both IL-1α and IL-1β were generated by
PCR from plasmids (kindly provided by Roy Black, Immunex Corp., Seattle, WA) that encoded the precursor cDNAs of IL-1α and IL-1β. PCR primers were designed such that restriction endonuclease sites were encoded either immediately 5' or 3' of the IL-1 protein coding sequences. 5' PCR primers for mature IL-1α and IL-1β cDNAs encoded restriction endonuclease sequences, followed by an ATG codon, and then sequences encoding bases 337–351 or 349–363 of IL-1α or IL-1β cDNAs, respectively, as described previously (40). cDNAs and retroviral vectors were digested with the appropriate enzymes, gel purified, and then ligated at 4°C overnight with T4 ligase (Boehringer Mannheim, Mannheim, Germany). Retroviral vectors pLXSN and pLPXPOX were provided by Dr. A. D. Miller (41). The pLXSH and pLPXPOX vectors were modified versions of these original vectors carrying the hygromycin and puromycin phosphotransferase genes, respectively (Fig. 5). Because pro-IL-1β protein was not produced from the above-described vectors, an alternative pro-IL-1β cDNA that encoded a 40-base 5' alfalfa mosaic virus untranslated leader sequence was kindly provided by Dr. Lee Gehrke of Harvard University (42) and was ligated into the pLXSN retroviral vector. These retroviral vectors were transfected into DH10B Escherichia coli, and ampicillin-resistant colonies were collected. Plasmid preparations from these colonies were digested with appropriate restriction endonucleases such that appropriately sized inserts (and where necessary, proper orientation) could be confirmed. Retroviral vectors were then transfected using calcium phosphate into PE501 murine fibroblast cells; viral supernatants were collected and then used to infect the primate specific packaging cell line, PG13. PG13 cells were selected with 2.5 mg/ml puromycin, 1 mg/ml G418, or 300 units/ml hygromycin. Resistant colonies were picked, and IL-1 production was assessed by ELISA (R & D Systems, Minneapolis, MN). Retrovirus-containing supernatants from IL-1-expressing clones were collected and then either used immediately or stored at -70°C until used. Viral supernatants were supplemented with 4 μg/ml hexamethylene bromide (Sigma Chemical Co., St. Louis, MO) and then incubated with pre-confluent 18-1153 cells at 37°C overnight. The next day, cells were split 1:2 and re-plated in plain keratinocyte-SFM (Life Technologies, Inc.). On the following day, selection was started in the presence of 100 units/ml hygromycin or 0.625 mg/ml puromycin or 100 mg/ml G418. Colonies were isolated after 10–12 days. IL-1 production was assessed by ELISA (R & D Systems). To produce 18-1153 cells expressing various combinations of IL-1α and IL-1β, cell lines were sequentially infected with a second viral supernatant after expression of the first IL-1 species had been confirmed.

RNA Preparation and Northern Analysis. RNA was prepared by the procedure of Chomczynski and Sacchi (43). Briefly, solution D (guanidium thiocyanate, sodium citrate, sarcosyl, and 2-mercaptoethanol) was added directly to monolayer cultures, and cells were removed with a cell scraper. Sodium acetate, phenol, and chloroform-isooamyl alcohol were added sequentially, and samples were incubated on ice for 15 min. Following centrifugation, the samples were taken through the various precipitations, and extractions were done as described (43), plus additional extractions with 1:1 phenol:chloroform and 4:1 chloroform:butanol. Finally, the aqueous portions of the samples were precipitated with ethanol and stored at -70°C. Ten μg of each sample were used in Northern blots that were performed as described previously (44). Probes used were those encoding precursor IL-1α and IL-1β cDNAs described previously.

Protein Preparation, ELISA Analysis, and Bioactivity Assays. Supernatants were prepared by incubating monolayer cultures overnight at 37°C with 5 ml of keratinocyte-SFM (Life Technologies, Inc.). The medium was collected and filtered through a 0.2 μm syringe filter (USA/Scientific) and then used in ELISA and bioassays. Cytosplasmaic proteins for ELISA analysis were prepared by resuspending 2 × 10^6 cells in 100 μl of a protein extraction solution consisting of 50 μl HEPES (pH 7.9), 250 μl KCl, 0.1 mm EDTA, 0.1 mm EGTA, 0.1% NP40, 0.4 mm NaF, 10% glycerol, and protease inhibitors (0.01 mg/ml pepstatin, 0.2 mg/ml Prefrac, 0.1 mg/ml aprotinin, and 0.005 mg/ml leupeptin; Sigma Chemical Co.) and incubating on ice for 30 min. Samples were spun at 14,000 rpm for 12–15 min in a tabletop Microfuge, and supernatants were collected. Protein samples were diluted with protein extraction buffer to a concentration that represented proteins from 10^6 cells/ml and tested for IL-1α and IL-1β by ELISA (R & D Systems). Cytosplasmic proteins for use in bioassays were prepared by resuspending 12 × 10^6 cells in 240 μl of a mild lysis solution composed of 20 mm Tris, 50 mm NaCl, 0.1 mg/ml aprotinin, and 0.005 mg/ml leupeptin in an Eppendorf tube. The Eppendorf tubes were then exposed to five or six 30-s bursts of sonication in a cup horn sonicator such that sterility of the samples was maintained. Lysis of cells was confirmed by microscopic inspection. Bioassays were performed at the Immunex Corp. (Seattle, WA) using an assay based on the ability of IL-1 to produce toxicity in the human melanoma A375 cell line (45).

Tumorigenicity Studies. Tumorigenicity of the various 18-1153, vector-infected 18-1153 and IL-1-expressing 18-1153 cell lines was assessed by s.c. injection in BALB/c nu/nu mice obtained from either Simon Laboratories (Gilroy, CA) or Charles River Laboratories (Temescola, CA). Cells were transplanted, counted on a hemocytometer, and then after centrifugation, resuspended in keratinocyte-SFM (Life Technologies, Inc.) such that 3 × 10^5 cells could be injected in 400 μl. After

![Fig. 5. Schematic representation of retroviral vectors used for IL-1 expression. * portions of vectors that are unique to pLXSN-derived expression vectors. AMV-UTL is a 40-bp alfalfa mosaic virus untranslated leader sequence that was added to the 5' end of the pro-IL-1β cDNA to increase translation of this protein.](https://image-url.com/image.png)
injection, mice were inspected twice weekly, and tumor areas were measured using calipers. Some mice were sacrificed and tumors were split, one-half being fixed for microscopic examination and the rest being used to establish cells in tissue culture. Cultures were established by mincing tumors finely and resuspending in keratinocyte-SFM containing collagenase (Worthington Biochemical, Freehold, NJ), penicillin-streptomycin, and fungizone and incubating overnight at 37°C. On the following day, tumor suspensions were washed in PBS, spun down, resuspended in 3 μl of trypsin, and incubated for 15 min at 37°C. This was followed by two more washes with PBS, and cells were then plated in keratinocyte-SFM. Two types of mixed tumor or transplant assays were also done. In the first, 3 × 10⁸ 18-115S cells were mixed with 3 × 10⁷ cells of either the vector-only infected cells (HPV) or nontumorigenic 18-115S cells expressing pro-IL-1α and mature IL-1β (αfβfM) and then injected into nude mice. In the other, nude mice first received injections over the right shoulder with 3 × 10⁸ cells of either HPV or αfβfM cells, and two weeks later, they were received injections over the left shoulder with 3 × 10⁸ 18-115S cells.

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References


