

The Induction of IL-6 and Gelatinase B by IL-1 in Mouse Cell Lines Transformed with Bovine Papillomavirus: Decreased Production in Tumorigenic Cells

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ABSTRACT

Six cell lines, that were cloned from murine C127 cells infected by bovine papillomavirus type 1 (BPV1), were found to differ in the degree of transformation *in vitro* and of tumorigenicity *in vivo*. In these cell lines the degree of tumorigenicity was inversely correlated with IL-6 induction by IL-1 β . Whereas the parental C127 cell line produced 15-30 U/ml of IL-6 spontaneously, none of the transformed cell lines produced significant levels of IL-6 constitutively. On induction by human IL-1 β the parental C127 cell line produced up to 300 U/ml of IL-6, whereas the fully transformed ID14 cell line failed to produce any. The less transformed cell lines produced lower yields of IL-1 β -induced IL-6, dependent on their degrees of transformation and tumorigenicity. Gelatinase B (96 kDa), a matrix metalloproteinase inducible by IL-1 β , was dose-dependently regulated in the parental C127 cell line and in the weakly transformed cell line T1c. These data suggest that transformation processes by BPV1 generally impair IL-1-regulated gene transcription. This impairment seems not to be located at the IL-1 β receptor level, since in all the cell lines studied the numbers and affinities of the IL-1 β binding sites were found to be comparable. This impairment seems not to be mediated by transformation-induced inactivation of the protein kinase C pathway since phorbol 12-myristate 13-acetate (PMA) induced IL-6 production equally well in all C127 cell-derived clones. It is suggested that BPV1 transformation can change the expression of host genes that might play a functional role in tumor immune surveillance and tumorigenicity *in vivo*.

INTRODUCTION

Papillomaviruses are small double-stranded DNA viruses, responsible for a variety of benign skin and mucocutaneous tumors such as warts and condylomas. Recently, certain types of human papillomaviruses (HPV) were found to be associated with dysplasias and carcinomas (1). Bovine papillomavirus (BPV) induces fibropapillomas in the natural host, and can, after a latency period, induce fibroblastic tumors in heterologous hosts, e.g., horses, mice, hamsters. BPV type 1 (BPV1) also transforms mouse primary cells and immortalizes cell lines

such as C127 and NIH3T3 *in vitro*. With the use of these *in vitro* cell systems two transforming genes, E6 (2,3) and E5 (4-6), have been identified. The E2 gene products, *trans*-acting proteins of papillomaviruses, can indirectly affect transformation efficiency by regulating the transcription of transforming genes (7).

Carcinogenesis is a multistage process initiated by various events such as mutations or the expression of newly introduced genes. Epidemiological evidence indicates that other factors, such as smoking in the case of cervical carcinoma (8) or UV-light in the case of *Epidermodysplasia verruciformis* (EV) (9),

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might be involved in malignant conversion of papillomavirus-associated tumors. In primary and immortalized cell lines, induction of cellular genes, such as oncogenes, has been shown to be required for full transformation of papillomavirus-induced tumors (10,11). Recently, the E5 gene of BPV1 has been reported to activate the cellular PDGF receptor (12) and to stimulate the activity of EGF and CSF-1 receptors leading to increased transformation (13). The E6 and E7 proteins, transforming genes of HPV16 and HPV18, have been demonstrated to bind the tumor suppressive proteins p53 (14) and the retinoblastoma gene product (15), respectively. Thus, the interaction of viral and cellular genes is important for full transformation of normal cells. The BPV1 E2 gene has also been shown to induce cellular proteins in C127 cells (16,17) and to activate several cytokine promoters such as those of IL-2, IL-3, and GM-CSF (18). This suggests that early gene products of papillomaviruses regulate the expression of cellular genes that play a role in host defense. The host immune system plays an important role in the rejection of tumors since some types of HPV-associated benign and malignant tumors are more prevalent in patients with impaired immunity (19). The infiltration of inflammatory cells into warts undergoing regression has also been demonstrated (20).

Among various cytokines, IL-1, IL-6, IL-8, and TNF are often produced in response to injury, inflammation, or viral infection (21–27). In this study the effects of IL-1 on the induction of such a cytokine (IL-6) and of a matrix degrading enzyme (gelatinase) were investigated in a series of BPV1-transformed mouse cell lines that showed different degrees of transformation *in vitro* and of tumorigenicity *in vivo*.

MATERIALS AND METHODS

Cell line and tissue culture

The mouse cell line, C127, derived from RIII mouse breast tissue, was obtained from the American Type Culture Collection. Cell lines, T1c, T2, T3c, and T4a were cloned from C127 cell cultures infected with BPV1 as described before (17). The T2 cell line was isolated from foci in which cells exhibited elongated spindle shapes. The cell lines, T1c, T3c, and T4a, were cloned from colonies formed in soft agar plates. The BPV1-transformed cell line, ID14, was kindly supplied by Peter M. Howley, National Cancer Institute, NIH Bethesda, MD. All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum (FCS).

Anchorage-independent growth

Triplicate 60 mm dishes were seeded with 5×10^4 cells in DMEM containing 0.35% agar (Bacto agar, Difco, MI) and 10% FCS. Colonies containing more than 20 cells were counted after 21 days.

Tumorigenicity in nude mice

Monolayer cells in the late log phase were harvested by trypsinization. Cells (5×10^6) suspended in 0.2 ml DMEM

were injected intramuscularly in the left leg of female NMRI (nu/nu) nude mice at 6 weeks of age. All mice were regularly examined for tumor development for at least 3 months following injection.

Induction and measurement of IL-6

BPV1-transformed cell lines were cultured in 24 multiwell plates at a concentration of 1.5×10^5 cells/ml in DMEM containing 5% FCS. After 24 to 48 h monolayers were washed 3 times with DMEM and treated for 48 h with or without human natural IL-1 β (22) or phorbol 12-myristate 13-acetate (PMA) in DMEM supplemented with human serum albumin (6 mg/ml). IL-6 activity was measured as hybridoma growth factor. Briefly, murine B-cell hybridoma (7TD1) cells were incubated with serial dilutions of culture fluids or of purified IL-6. After 4 days colorimetric staining was used as a parameter for cell density (28). One U/ml of IL-6 (1 pg/ml) corresponds to half maximal cell growth.

Assay of gelatinase activity

Gelatinase activity in cell culture supernatant was determined by SDS-PAGE zymography and quantified by scanning densitometry as reported previously (29,30).

IL-1 binding assay

125 I-labeled human IL-1 β was purchased from Amersham International, Buckinghamshire, UK. The specific activity was 3200 Ci/mmol. For binding experiments 5×10^5 cells were inoculated in 6 multiclustered plates. After 48 h incubation in DMEM containing 5% FCS, the cells were washed with DMEM and incubated with 125 I-labeled IL-1 β at different concentrations with or without 50-fold excess of unlabelled human natural IL-1 β in 0.5 ml of DMEM containing BSA (1 mg/ml). After 2 h incubation at 22°C with gentle continuous shaking, the supernatants and first washes were collected and counted for the measurement of free IL-1 β . The monolayer of cells was washed three times with ice-cold phosphate-buffered saline (PBS), dissolved into 1 ml of 1% SDS solution and counted for the measurement of bound IL-1 β . The specific binding was calculated by subtraction of the amount of labeled IL-1 β bound in the presence of excess unlabeled IL-1 β from that bound in the absence of unlabeled IL-1 β .

RESULTS

Tumorigenicity of transformed cells in nude mice

Several clones from C127 cells transformed by BPV1 were isolated. Transformants showed different degrees of expression of the transformed phenotype as monitored by saturation density, doubling time, growth in medium with a low serum concentration, and colony-forming efficiency (17). Six different cell lines were tested for their anchorage-independent growth in soft agar (Table 1). The results obtained were consistent with those described earlier (17) except that the T2 cell line produced more foci than before. These cell lines were injected into nude

TABLE 1. TUMORIGENICITY OF C127 CELL LINES TRANSFORMED BY BPV1

Cell line	Copy number of virus genome ^a	Number of colonies in soft agar ^b	Tumorigenicity in nude mice	
			Tumor formation	Tumor size ^c (mm ³)
C127	0	0	0/6	—
T1c	H	50 ± 30	0/4	—
T2	L	130 ± 50	4/4	5.0 ± 0.8
T3c	L	1700 ± 350	3/3	15.1 ± 3.8
T4a	H	3200 ± 570	4/4	30.8 ± 5.4
ID14	H	5400 ± 710	6/6	52.6 ± 3.7

^aL, 5 copies per cell; H, 50 copies per cell.

^bThe data represent the mean ± SD of three independent experiments.

^cFifty days after inoculation. The data represent the mean ± SD.

mice in order to characterize the tumorigenic properties *in vivo*. One week after intramuscular injection of ID14 cells, small swellings appeared at the site of injection. Approximately 3 weeks after inoculation, fast growing solid tumors were recognized. In one of the mice injected with ID14 cells, metastasized tumors were detected in the liver. The cell lines T3c and T4a, representing the moderately transformed group, also gave rise to fast tumor development but in all cases without evidence for metastasis. Smaller tumors were also induced by the T2 cell line. The growth rate of this cell line was very slow compared to that of the other 3 cell lines. In the nude mice inoculated with the untransformed C127 cell line or the lowly transformed T1c cell line, small swellings were also found 1 week after inoculation but no tumor development occurred over a period of 2 months. Histological examination of tumors developed in nude mice revealed a fibrosarcoma-like phenotype (data not shown). Each cell line retained its original phenotypic characteristics as observed in *in vitro* cell cultures; the T2 cell line showed a more epithelial culture pattern and the T3c, T4a, and ID14 cell cultures contained spindle and fibroblastic type cells.

Induction of IL-6

We have previously shown that production of IL-6 by human and animal cells is regulated by several mediators of which IL-1 β is the most potent physiological one (23,25,31). In view of the immunomodulating effects of IL-1 and IL-6, the constitutive as well as the induced amounts of IL-6 by the BPV1-transformed cell lines were investigated. In contrast to the untransformed parental cell line C127, which spontaneously produced low levels (15 U/ml) of IL-6, none of the cell lines transformed with BPV1 constitutively produced significant levels of IL-6 (Fig. 1). C127 cells responded well to the stimulation by IL-1 β and secreted about 300 U/ml IL-6 into the culture medium. Concentrations as low as 0.1 U/ml of IL-1 β were able to induce a significant increase in IL-6 levels. The fully transformed ID14 cell line did not respond to even the highest concentration of IL-1 β (100 U/ml). The T1c cell line, showing a low degree of transformation but no tumorigenicity, also produced IL-6 after IL-1 treatment. However, the dose of IL-1 β needed for effective inductions was higher, whereas the amount of IL-6 produced was less than that seen in C127 cells. The

maximal amount of IL-6 induced in BPV1-transformed cells significantly decreased as their tumorigenicity increased.

To test whether transformed cells lost their ability to express the IL-6 gene, cells were treated with PMA, a strong inducer of IL-6. Except when stimulated with the highest dose (100 ng/ml), all cell lines produced comparable amounts of IL-6 in a similar dose-dependent manner (Fig. 2).

Induction of gelatinase

Recently, we and others found that the synthesis of gelatinase is regulated by IL-1 β in monocytes (32,33) as well as transformed macrophages/monocytes (34) and in fibrosarcoma cells (35). Therefore, the induction of gelatinase B (96 kDa) by IL-1 β was measured to investigate whether another IL-1-induced gene product is impaired on BPV-transformation (Fig. 3). We could detect gelatinase B in the BPV-transformed cell lines. Except in the T1c cell line, the levels of the constitutively produced gelatinase B were very low compared to those of the

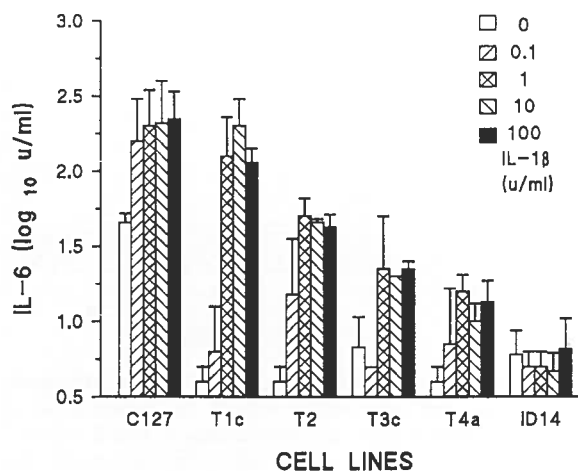


FIG. 1. Production of IL-6 by BPV1-transformed mouse cell lines treated with IL-1 β . Confluent monolayers of each cell line were treated with serial dilutions of human natural IL-1 β . The results represent the mean ± SD of three independent experiments.

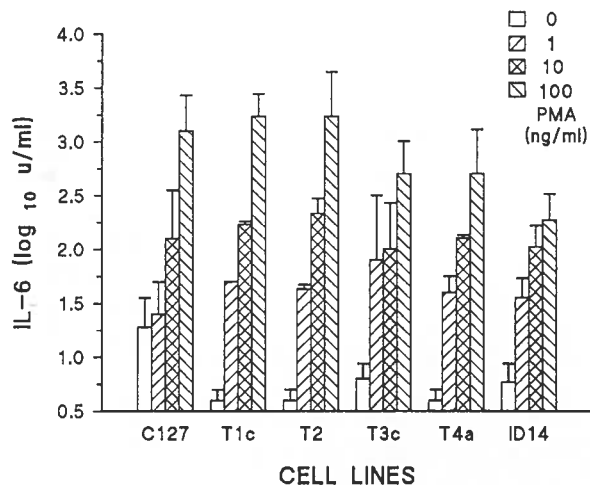


FIG. 2. Production of IL-6 by BPV1-transformed mouse cell lines treated with PMA. Confluent monolayers of each cell line were treated with serial dilutions of PMA. The results represent the mean \pm SD of three independent experiments.

parental C127 cell line. IL-1 β could induce the gelatinase B activity only in the untransformed C127 cell line and the weakly transformed T1c cell line. Thus, the profile of the gelatinase B induction by IL-1 β in the BPV1-transformed mouse cell lines was found to be similar to that of IL-6 induction. This suggests a rather general impairment of IL-1 stimulated signal transduction by BPV1 transformation.

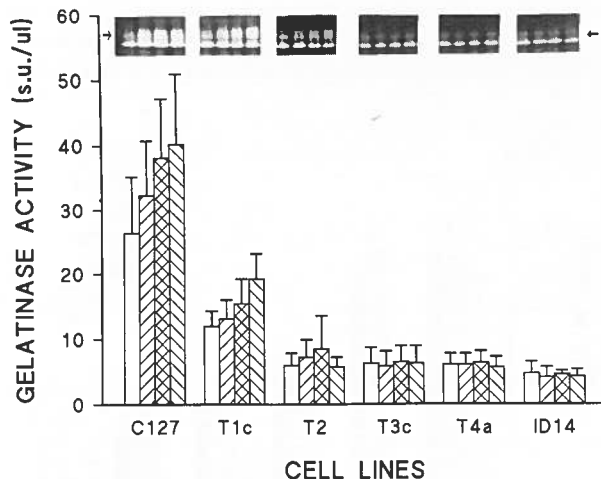


FIG. 3. Production of gelatinase B (96 kDa) by BPV-transformed mouse cell lines treated with IL-1 β . Supernatants of confluent cultures of different cell lines treated with IL-1 β for 2 days were analyzed for gelatinase activity by zymography (inset). Arrows indicate the position of the 96-kDa gelatinase. The enzyme activity was determined by densitometric scanning analysis and expressed as scanning units (s.u.). Histograms corresponding to different doses of IL-1 (0, 1, 10, 100 U/ml) are hatched as for PMA doses in Fig. 2. The results represent the mean \pm SD of four independent experiments.

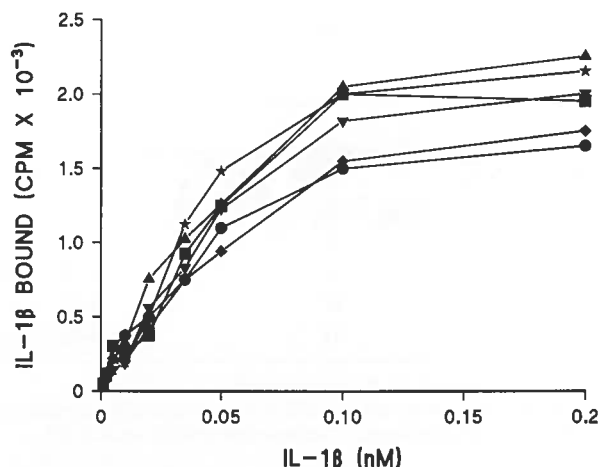


FIG. 4. Saturable specific binding of ¹²⁵I-labeled IL-1 β to BPV1-transformed mouse cell lines. (●) C127; (▲) T1c; (★) T2; (■) T3c; (▼) T4a; (◆) ID14. Monolayers of each cell line were incubated in DMEM containing serial dilutions of human recombinant ¹²⁵I-labeled IL-1 β for 2 h at 20°C. Specific binding was corrected for nonspecific binding in the presence of 50-fold excess unlabeled IL-1 β .

Binding of ¹²⁵I-labeled IL-1 β

To assess the lower sensitivity of BPV1-transformed cells to IL-1 β , the binding ability of IL-1 β to these cell lines was tested. Of the total binding, nonspecific binding was approximately 30% in all cell lines tested when measured by adding a 50-fold excess of unlabelled human natural IL-1 β . Specific binding of ¹²⁵I-labeled IL-1 β to 6 cell lines is shown in Fig. 4. The binding was saturable and half maximal binding occurred at 3–4 $\times 10^{-11}$ M. The number of binding sites calculated from this data by Scatchard analysis are shown in Table 2. The number of binding sites varied and was highest in T2 cells and lowest in T3c cells. ID14 cells that are insensitive to the IL-6 inducing activity of IL-1 β have 2200 binding sites per cell. In conclusion, we found no correlation between the sensitivity of cells to IL-1 β action and the number of IL-1 β -binding sites.

TABLE 2. IL-1 RECEPTORS ON C127 CELL LINES TRANSFORMED BY BPV1

Cell line	Binding of IL-1 β (K_d)	Number of molecules bound per cell
C127	6.2 \pm 0.31 $\times 10^{-10}$ ^a	1736 \pm 245 ^a
T1c	6.1 \pm 0.25 $\times 10^{-10}$	2170 \pm 384
T2	1.4 \pm 0.09 $\times 10^{-9}$	2697 \pm 408
T3c	5.8 \pm 0.39 $\times 10^{-10}$	1085 \pm 283
T4a	1.2 \pm 0.08 $\times 10^{-9}$	1550 \pm 180
ID14	1.1 \pm 0.07 $\times 10^{-9}$	2201 \pm 284

^aThe data represent the mean \pm SD of three independent experiments.

DISCUSSION

The use of C127 cells as a substrate for transformation by BPV1 has allowed an initial comparison of alterations in cellular phenotypes induced by the oncogenic virus. Using this system it has been reported that BPV-transformed primary mouse fibroblasts (36) and rat embryo fibroblasts (37) showed different degrees of transformation and tumorigenicity in nude mice. As we reported previously (17), BPV1-transformed C127 cell clones can be grouped into three classes on the basis of their degree of transformation *in vitro* as quantified by cellular growth rate, saturation density, growth in low concentrations of serum, and anchorage independency. Tumorigenicity of these different cell lines *in vivo* was in agreement with *in vitro* observations (Table 1). The differences in tumorigenicity might reflect the influence of cellular rather than viral factors, since the expression of viral mRNA in different transformed cell lines was in the same order of magnitude in all cell lines tested. Paradoxically, the T1c cell line contained a high copy number of the virus genome while the more tumorigenic cell lines, T2 and T3c, contained only low copy numbers (Table 1).

IL-1 is a cytokine that participates in the regulation of inflammatory reactions, immune responses, and hematopoiesis (21). IL-6 is a cytokine released by different types of cells and plays a crucial role in the immune response through activation of B and T cells (24). Expression of the IL-6 gene is inducible by many inflammation-regulatory substances including IL-1 and TNF (23). IL-6 induction by IL-1 β in C127 cell lines transformed by BPV1 was well correlated with the tumorigenicity of these cell lines. The nontransformed C127 cell line produced the highest amounts of IL-6 while transformed cells produced less as their tumorigenicity increased (Fig. 1).

Gelatinase B is a marker enzyme inducible by IL-1 β in various cell types including transformed cells. Since in our study both an IL-1 inducible cytokine as well as an IL-1 regulated metalloproteinase were affected by the BPV1 transformation, it looks as if the transformation process impairs IL-1 mediated gene transcription in a general way. This impairment seems not to be mediated by changes in IL-1 β receptor numbers or affinities (Fig. 4 and Table 2) and not by impairment of the protein kinase C pathway (Fig. 2).

The level of expression of IL-1 receptors among different C127-derived cell clones varied, but remained within the range of that in other cell types (38). Induction of IL-6 in the cell clones in response to IL-1 did not correlate with the number of IL-1 binding sites nor with the magnitude of the dissociation constant. The concentration of IL-1 β corresponding to half-maximal binding (30 pM) (Fig. 4) was not in the same range as the one needed for half-maximal stimulation of IL-6 in the C127 cell line (0.02 pM) and the T1c cell line (0.5 pM) (Fig. 1). It is possible that a small number of biologically active receptors is expressed in nontransformed C127 clones. The presence of high and low affinity receptors for IL-1 on mouse T cell lines has been reported by Lowenthal and Macdonald (39). Recently, two IL-1 receptors which showed different affinity against IL-1 β have been characterized by molecular cloning analysis (40).

Inflammatory cells may have a role in surveillance against papillomavirus infection and subsequent development and pro-

gression of papillomavirus-induced tumors. Indeed, infiltration of macrophages occurs in both the epithelium and stroma of HPV-infected tissues and CIN lesions (41). Also, infiltration of NK cells was found in the subepithelial stroma of HPV-infected tissues (42). Furthermore, regression of flat warts starts with a dramatic inflammation caused by a mononuclear cell infiltration (43). Finally, killing of HPV16-transformed cells is mediated by NK-cells (44) and macrophages (45,46). In response to viral infection, these inflammatory cells have been shown to secrete a wide range of cytokines, including IL-1, IL-6, and chemotactic factors (27). Therefore, it is possible that the highly tumorigenic cell lines transformed by BPV1 can escape the host defense network through resistance to IL-1 action.

IL-1 and TNF were reported to induce a rapid and transient accumulation of cyclic AMP (47). Recently, the cytokines IL-1 and TNF were shown to lead to activation of the NF- κ B-like proteins or NF-IL-6 that bind to specific motifs on the IL-6 gene (48). PMA induced IL-6 almost equally well in all cell lines in this study (Fig. 2), suggesting that the difference of sensitivity to IL-1 reflects the presence of a signal transduction system different from that used by PMA.

Our results demonstrate that the BPV1-transformed cell lines contribute a useful model system to elucidate alterations in signaling pathways caused by papillomaviruses and also further exemplify that cytokines/growth factors might influence tumor-virus-induced tumorigenicity *in vivo* and vice versa.

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