The Autonomous Growth of Human Papillomavirus Type 16-Immortalized Keratinocytes Is Related to the Endothelin-1 Autocrine Loop

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Some human papillomaviruses (HPVs) such as HPV type 16 (HPV16) and HPV18 are involved in cervical carcinomas, and they can immortalize and transform keratinocytes. Endothelin-1 (ET-1) is produced in keratinocytes and has been shown to act through ETA receptors as an autocrine growth factor for keratinocytes. This study examines whether HPV16 alters the ET-1-mediated autocrine loop in human keratinocytes, providing a selective growth advantage for transformed cells. ET-1 is released in similar amounts from normal and HPV-transfected keratinocytes. All HPV-transfected cell lines express high-affinity ETA receptors. A two-fold increase in ET-1 binding sites is present in HPV16-immortalized keratinocytes, and this effect seems to be linked to the overexpression of mRNA for this receptor rather than to differences in the surface/internalized ratio of the receptors. ET-1 induces significant increases in [3H]thymidine incorporation and cell proliferation. Furthermore, HPV-transfected keratinocytes can proliferate in the absence of any growth factor added to the growth medium, and the ETA receptor antagonist BQ123 prevents this proliferation. These data suggest a new mechanism in the growth control of HPV-transformed cells mediated by the upregulation of ET-1 autocrine loop.

A subset of human papillomaviruses (HPVs), including HPV types 16 and 18 (HPV16 and HPV18), is associated with cervical cancers (15, 48). Transfection of keratinocytes and exocervical cells with HPV16 DNA induces their immortalization and transformation in culture (2, 26). The E6 and E7 viral genes play an important role in this process through the interaction with the cellular oncogenes p53 and Rb (12, 38). Although this interaction appears to be essential for intra cellular transformation (47), other alterations could be relevant in this process. HPV transformation induces changes of keratinocyte responsiveness to endogenous growth factors leading transformed cells to have a reduced requirement for growth factors (8, 25). It has been reported that autocrine growth factors are essential for the rapid proliferation of some transformed cells (23). In addition, a growth advantage for transformed cells appears to be the overexpression of growth factor receptors that in general is linked to amplification and/or rearrangement of the receptor genes (14, 28, 40). Recent reports indicate that HPV16 immortalization of human keratinocytes (36) and exocervical epithelial cells (35) may cause a specific increase in the sensitivity to growth stimulation by epidermal growth factor (EGF), possibly increasing the level of EGF receptors (EGFRs). This effect seems to be linked to the expression of the oncoprotein E5 which could inhibit the degradation of the receptor following the internalization of the receptor-ligand complex (36). Keratinocytes can produce growth factors other than EGF that influence the neighboring cells, namely, nerve growth factor (NGF) (11), basic fibroblastic growth factor (16), and tumor growth factor alpha (10), and some of them can stimulate cell proliferation through an autocrine loop.

Endothelins (ETs) are a family of three vasoactive peptides, termed ET-1, ET-2, and ET-3, with profound cardiovascular, mitogenic, and neuroregulatory functions (9, 37, 44). ETs induce their biological actions through at least two major receptor subtypes that belong to the family of G-protein-coupled receptors: a selective ETA receptor, which binds ET-1 and ET-2 with high affinity and ET-3 with low affinity; and a non-selective ETB receptor that binds all ET isopeptides with equal affinity (3, 29, 30). ET-1 is produced by a variety of human cancer cells (22, 24, 43) and could be involved in the pathogenesis of certain types of cancer through an autocrine loop. ET-1 is also secreted by human keratinocytes (19) and is involved in the regulation of melanocyte proliferation and pigmentation (42). Recently, we reported that ET-1 can act through ETA receptors as an autocrine growth factor for primary keratinocytes (4). The present study examines whether HPV16 alters the ET-1-mediated autocrine loop, providing a selective growth advantage for HPV-transformed cells. We have determined and compared the ET-1 binding sites and binding affinity, the secretion of ET-1, and the ET-1-mediated proliferative effect in normal human keratinocytes and in cell lines transformed by the complete genome or the E6 and E7 sequences of HPV16.

MATERIALS AND METHODS

Cell culture. Normal human epidermal keratinocytes (NHKEK) were isolated from child foreskins (kindly provided by the Division of Plastic Surgery, “Bambino Gesù” Pediatric Hospital, Rome, Italy) as described by Pirisi et al. (26) except that the epidermis was separated from the dermis by overnight digestion with 0.25% trypsin (GIBCO). Purity of the culture was confirmed by cuboidal morphology and positive immunostaining with antikeratin and/or anti-involucrin antibodies. HKc/HPV16 (26) and E6-E7/HPV16 cell lines (gift of J. di Paolo, National Institutes of Health, Bethesda, Md.) were human keratinocytes immortalized by the complete genome of HPV16 and the E6-E7 open reading frame.
alone, respectively. HK168 was an HPV16-transfected cell line recently established in our laboratory by lipofection with the complete genomic sequence of HPV16 and serum selection as described by Schlegel et al. (31). Thus far, cell line HK168 has shown an expanded life and a resistance to cell differentiation (37a). All experiments were maintained in a conditioned medium without serum consisting of modified MCDB 153-LB medium supplemented with hydrocortisone (0.2 μM), transferrin (10 μg/mL), insulin (5 μg/mL), triiodothyronine (10 nM), CaCl₂ (0.09 mM), EGFR (5 ng/mL), and bovine pituitary extract (35 to 50 μg of protein/ mL).

Transfection. NHEK were transfected by lipofection with plasmid pMH16Vd, consisting of a head-to-tail HPV16 DNA dimer cloned into plasmid pMMTneo (gift of J. di Paolo). Briefly, 10 μg of pMMTneo or pMH16Vd mixed to 45 μg of DOTMA (Boehringer Mannheim) in a final volume of 200 μL was added dropwise to keratinocytes grown to subconfluence in 100-mm diameter petri dishes. After 24 h, the medium was replaced; at confluence, the cells were seeded on plastic wells for the receptor binding study.

ET-1 extraction. Keratinocytes were grown in 75-cm² plastic flasks until 90% confluence (2 × 10⁶ cells) and then growth factor starved for 24 h. Each culture flask was subjected to further incubation for various time intervals to obtain the conditioned medium used for subsequent analysis. Cells were counted in a hemocytometer, and the viability was 80 to 90% by trypan blue dye exclusion. For chromatographic analysis, conditioned media were acidified with 0.1% trifluoroacetic acid, and the supernatant was applied to Sep-Pak C₁₈ cartridges (Water Associates, Milford, Mass.). After elution with 2 mL of 60% acetonitrile–0.1% trifluoroacetic acid, the HPLC fraction was analyzed for ET-1 by reverse phase radioimmunoassay (RIA). The recovery of synthetic ET-1 by the extraction procedure was 78%.

RIA. The hophylized ET-1 samples were reconstituted in an assay solution containing 0.1 M phosphate buffer (pH 7.4) containing 0.05 M NaCl, 0.1% bovine serum albumin (BSA), 0.1% Triton X-100, and 0.01% sodium azide. Samples were equilibrated for 24 h at 4°C with an anti-ET-1 antibody (Peninsula Laboratories, Belmont, Calif.) followed by the addition of 20,000 cpm of [³²P]ET-1 (2.200 Ci/mmol; DuPont NEN). After incubation for an additional 48 h, free and antibody-bound tracers were separated by the addition of sheep antiserum to rabbit immunoglobulin and incubated for another 2 h at room temperature. The cross-reactivities of the antisera for ET-related peptides (relative molar affinity of ET-1, considered 100%) were as follows: ET-1, 7%; ET-2, 7%; ET-3, 7%; and bigET-1, 17%. The sensitivity of the ET-1 RIA was 1.5 pg/tube, and the 50% intercept was 36 pg/tube. The intra- and interassay variations were 8 and 13%, respectively.

Binding studies. Keratinocytes were cultured in six-well plates until confluent (8 × 10⁵ cells/well) and then growth factor starved for 24 to 48 h. After a wash with assay buffer composed of Hanks balanced salt solution, 0.2% BSA, and 100 μg of bacitracin per mL, cells were incubated in 500 μL of assay buffer containing 40 pM [³²P]ET-1 for time periods ranging from 0 to 180 min at temperatures of 4, 22, and 37°C. For equilibrium binding studies with [³²P]ET-1, cells were incubated at 37°C for 60 min with increasing concentrations of the radioactive tracer in the presence or absence of an excess (1 μM) of unlabeled ET-1 (Peninsula Laboratories). For binding inhibition studies, keratinocytes were incubated with tracer and ET-related peptides, including ET-1, ET-2, ET-3, sarafotoxin 6c (S6c), and BQ123 (Peninsula), as well as the unrelated peptide NGF (gift of D. Mercart, Consiglio Nazionale delle Ricerche, Rome, Italy). At the end of incubation, cells were washed three times with cold phosphate-buffered saline solution containing 0.2% BSA, then solubilized with 1 N sodium hydroxide, and analyzed for bound radioactivity in a γ spectrometer. The nonspecific binding, determined in the presence of 10⁻⁷ M unlabeled peptide, was 5% of the added radioligand.

The number of binding sites was determined by using the LIGAND program.

Receptor internalization. To measure the surface-bound and internalized [³²P]ET-1, the cell monolayers were rinsed three times at 4°C with 0.15 M NaCl after the radiolabeled ET-1 binding, and then ice-cold 0.2 N acetic acid (pH 2.5) containing 0.5 M NaCl was added for 2 min. The acid-salt wash was removed, the radioactivity in it was counted; the cells were scraped in 0.1 N NaOH, and the remaining cell-associated radioactivity was quantified. [³²P]ET-1 removed by the acid-salt rinse was considered to be present on the cell surface, and [³²P]ET-1 resistant to the acid-salt wash was considered to be internalized.

Reverse transcription-PCR (RT-PCR). Total RNA was extracted and purified from cell cultures by a commercial kit (RNAzol; Biotec Laboratories Inc., Houston, Tex.) that utilizes the guanidinium thiocyanate method. One microgram of RNA was reverse transcribed to cDNA and amplified by using an RNA PCR kit (Perkin-Elmer Co.) according to the manufacturer’s instructions, with specific primers for the ETₐ receptor mRNA, i.e., upstream primer 5'-CATCTGTTGAGTGTGTTAAT-3' (nucleotides 38 to 57) and downstream primer 5'-GGAGACATCCTGACCATCACTG-3' (nucleotides 386 to 405), as reported by Adachi et al. (1). Briefly, reverse transcription was carried out at 42°C for 15 min in the presence of downstream primers. The reaction was stopped by incubation at 99°C for 5 min and then chilled in ice. The cDNA was amplified with 2.5 U of AmpliTaq (Roche) in the presence of upstream and downstream primers for the ETₐ receptor. β-Actin primers were included as an internal control. In all experiments, two control reactions, one containing no mRNA and another containing no reverse transcriptase, were included. The specificity of the amplified product was verified by the presence of products of the expected size.
Receptor number and internalization of ET-1 receptor in HPV16-immortalized keratinocytes. As in normal human keratinocytes, all cell lines that produced ET-1 peptide expressed high-affinity receptors. Specific binding was rapid and was time and temperature dependent. Specific binding was maximal after 60 min of incubation at 37°C and remained constant up to 90 min. At 22 and 4°C, the maximum specific binding was obtained at 120 min and was only 40% of that observed at 37°C. Subsequent binding studies were performed at 37°C for 60 min (data not shown). [125I]ET-1 binding to normal and HPV16-transfected cells was saturable, and a twofold increase in ET-1 binding was present in keratinocytes immortalized by HPV16 genomic sequences (Fig. 2). Radioligand binding was performed at 37°C for 1 h under conditions in which the receptor number could be overestimated, considering that the internalization rate of this receptor is quite rapid. Nevertheless, binding studies revealed that the variation in ET-1 binding capacity was probably accounted for by an increased number of receptors in keratinocytes immortalized by the complete genome of HPV16. Normal keratinocytes isolated from different donors showed no significant differences in ET-1 receptor number. Only cell lines transfected by the complete genome of HPV16 showed an overexpression of receptors with respect to normal keratinocytes, whereas the E6-E7/HPV16 cell line had a number of receptors in the range of that for normal keratinocytes (Table 1).

Competition for [125I]ET-1 binding to the HKc/HPV16 cell line by unlabeled ET-1, ET-2, ET-3, NGF, the selective ETb receptor agonist S6c (37), and the ETA-selective antagonist BQ123 (12) is shown in Fig. 3. The maximal 50% inhibitory concentrations of ET-1 and ET-2 were 10–8, 1.2 × 10–10 M, respectively, whereas those of BQ123, ET-3, and S6c were 1.1 × 10–9, 5.2 × 10–8, and >10–6 M, respectively (Fig. 3). The specificity of binding was indicated by the inability of the unrelated peptide NGF to inhibit the binding of [125I]ET-1. These data, showing differences in the displacement capacity of ET-1 and ET-2 compared with ET-3 and S6c, suggest that the predominant receptor present in these cells was the ETA subtype.

It has been suggested that HPV can alter the proportion between surface and internalized EGFR through the action of the E5 protein and that this protein can increase the recycling of the same receptor to the cell surface (36). To determine the effect of HPV16 sequences on the rate and extent of ET-1 receptor internalization, the intracellular and surface-bound [125I]ET-1 were monitored over time in HKc/HPV16 and NHEK cell lines. The specific cell-bound radioactivity reached a steady state after 60 min of incubation at 37°C. Such a steady state represents the equilibrium between [125I]ET-1 binding-dissociation to its receptors and endocytosis of the receptor-ligand complex. Internalization of receptor-bound [125I]ET-1 occurred rapidly, and the rate and extent of endocytosis were dependent on incubation temperature and time. At 37°C, acid-sensitive binding reached an equilibrium after 30 min, and internalization began to contribute to the total binding after 15 min of incubation. Measurable endocytosis was observed at 8°C and was half-maximum at 22°C in cells exposed to [125I]ET-1 for 2 h in a range of incubation temperatures. No differences in the surface/internalized ratio of the ET-1 receptor were found in HPV-immortalized and normal keratinocytes, indicating that the number of total receptors, rather than their distribution, is altered; even the rate of internalization was the same, with 90% of radioligand internalized at 30 min (data not shown).

Receptor number in transfected keratinocytes. Comparisons of receptor number on immortalized keratinocytes were hampered by the difficulty of using the parental keratinocytes as

![FIG. 2. Saturation binding of [125I]ET-1 to NHEK (■), HKc/HPV16 (▲), and HK168 (●) cell lines. Increasing concentrations of [125I]ET-1 were added to cells in triplicate in the absence (total binding) or presence (nonspecific binding) of unlabeled ET-1 (1 μM).](image)

![FIG. 3. Inhibition of [125I]ET-1 binding by ET-1-related peptides. HKc/HPV16 cells were incubated in the presence of 40 pM [125I]ET-1 and increasing concentrations of ET-1-related or unrelated peptides. Binding is expressed as percentage of bound radioligand in the absence of unlabeled peptide; each point is the mean of data from three experiments.](image)

### TABLE 1. ET-1 receptor number in normal and immortalized human keratinocytes

<table>
<thead>
<tr>
<th>Cell line</th>
<th>No. of receptors/cell†</th>
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<tbody>
<tr>
<td>NHEK(296)</td>
<td>1,626 ± 70</td>
</tr>
<tr>
<td>NHEK(596)</td>
<td>1,462 ± 56</td>
</tr>
<tr>
<td>NHEK(996)</td>
<td>1,598 ± 68</td>
</tr>
<tr>
<td>E6-E7/HPV16</td>
<td>1,735 ± 65</td>
</tr>
<tr>
<td>HKc/HPV16</td>
<td>2,833 ± 120</td>
</tr>
<tr>
<td>HKc/HPV16</td>
<td>4,065 ± 127</td>
</tr>
</tbody>
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† Mean ± SD of three experiments, each performed in triplicate.
‡ Healthy donor number.
* P < 0.005 versus NHEK(296).
** P < 0.001 versus NHEK(296).
controls because of their limited lifetime. Moreover, long-term culture of cells could lead to alterations in some biological characteristic due to spontaneous mutation and/or growth selection over time. To overcome this problem, primary keratinocytes were transfected with the genomic sequences of HPV16 and with the vector alone. Shortly after transfection, without any selection of cell cultures, specific binding sites for $[^{125}I]$ET-1 were measured. In this way, the behavior of normal keratinocytes could be compared with that of transfected cells. The number of receptors per cell in the HPV16-transfected keratinocytes ($3,978 \pm 142$ [mean ± standard deviation {SD}]) was twofold greater than that in the controls ($1,504 \pm 82$). However, we estimated through the use of β-galactosidase activity staining in the presence of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) that only 30% of cells were transfected in this manner.

Expression of mRNA for ET$_A$ receptor subtype. Data from binding studies suggested that an increased number of binding sites was determined by overexpression of the specific receptor. To verify and confirm these results, mRNAs from all cell lines were extracted, and specific messengers for ETA receptor were reverse transcribed and amplified by RT-PCR. A single DNA fragment of the expected size (367 bp) which hybridizes with the internal oligonucleotide probe was amplified in all cell lines. The semiquantitative analysis of the amplified products showed only in HKc/HPV16 and HK168 keratinocytes a statistical significant increase in the expression of mRNA for ETA receptor with respect to controls (Fig. 4). These data correlated perfectly with those from binding experiments, thus confirming that cells immortalized by the complete genome of HPV16 expressed higher levels of specific receptors for ET-1.

Proliferative effects of ET-1 in HPV16-immortalized keratinocytes. Assays for ET-1-induced mitogenic responses were performed on HPV16-immortalized keratinocytes. $[^{3}H]$thymidine incorporation in HKc/HPV16 cells was stimulated by ET-1 in growth factor-free medium in a dose-dependent manner, with a significant increase with respect to normal cells (Fig. 5). Since ET-1 stimulated mitogenic responses in immortalized keratinocytes, we explored the possibility that ET-1 could act as an autocrine growth factor by coincubating the cells with ET-1 and the ETA receptor antagonists BQ123 and BQ610 (20). In this experiment, the stimulatory action of 100 nM ET-1 on $[^{3}H]$thymidine incorporation by HKc/HPV16 cells was completely inhibited in presence of 100 nM BQ123 and BQ610 (Fig. 6). The selective ET$_B$ agonist S6c failed to stimulate thymidine incorporation and did not affect the stimulatory action of ET-1; moreover, the selective ET$_B$ antagonist BO788 (21) failed to decrease the response to 100 nM ET-1 (Fig. 6). Taken together, these data indicate the presence of an ET-1 autocrine mitogenic signalling in HPV16-immortalized keratinocytes and confirm that this mitogenic signalling is mediated by the ETA receptor subtype. Furthermore, to establish whether endogenous ET-1 produced by the immortalized keratinocytes exerted a similar pro-
that HPV16 immortalization can lead to an increase in the autocrine proliferative response of keratinocytes to endogenous ET-1 stimulation. This vasoactive peptide is a potent growth factor for several cell lines (9). ET-1 is produced by human cancer cell lines, and it has been suggested that ET-1 may modulate in vivo the growth of stromal cells surrounding tumor cells and/or the growth of the cancer cells themselves, through paracrine or autocrine mechanisms. The role of ET-1 as an autocrine growth factor was reported for cell lines derived from ovary (5, 6) and HeLa (34) cells; HeLa is a cervical carcinoma-derived cell line in which HPV18 DNA sequences are present and transcriptionally active (32). Recently, we demonstrated that in normal keratinocytes, ET-1-induced DNA synthesis is mediated by the ETA receptor subtype and that the endogenously produced ET-1 promotes the autocrine proliferation of cells (4).

The HPV16-immortalized cell lines produce significant amounts of ET-1, as evidenced by the time-dependent accumulation of the immunoreactive peptide. The levels of ET-1 production by HPV16-transfected keratinocytes are similar to those of normal keratinocytes, which are within the biological range observed in other human cells such as normal breast epithelial cells (7), ovarian (5, 6) and breast (43) cancer cell lines, and several tumor cell lines (22). All HPV16 cell lines that produce ET-1 express receptors for ET-1. However, only cell lines immortalized by the complete genome of HPV16 show an increase in the number of this receptor on the cell surface. The presence of slight variations in the number of ET-1 receptors in normal keratinocytes from different donors validates the two-fold increase showed by HPV16-transfected cells. The relative potency of ET-1 and ET-2 compared with ET-3 and S6c for inhibition of 125I-labeled ET-1 binding indicates that as in normal keratinocytes, the predominant endothelin receptor in these cells is of the ETA subtype.

An important question concerns the mechanism by which ETA steady-state protein levels are elevated. The increased levels of ET-1 receptor, obtained in transient transfections of primary keratinocytes with genomic HPV16, exclude the possibility that long-term cultivation of immortalized keratinocytes can produce this biological alteration. In transient transfections, only 30% of the cells were transfected, which indicates that the number of ET-1 receptors is largely underestimated. This discrepancy in the number of ET-1 receptors between stable and transiently transfected cells could be due to a higher activity of viral transcripts in the early stage of transfection when the viral sequences are not integrated into the host genome. Thus, some viral protein could be involved in the overexpression of the ETA receptor. E5 gene products of HPV16 increase EGFR numbers in human keratinocytes and affect downregulation of the EGFR, primarily by increasing the number of receptors which recycle back to the cell surface after ligand binding (36). In the case of the ETA receptor, our data showing no significant difference in receptor recycling between normal and HPV16-transfected keratinocytes suggest that E5 is not involved in the upregulation of expression of this receptor, at least in the way shown for EGFR. Nevertheless, E5 could be involved in the amplification of ET-1 signal transduction as already reported for EGF (46). The E5 gene of HPV16 can induce cell proliferation in growth factor-free media in the presence of ET-1, and it can stimulate ET-1-induced DNA synthesis to the same extent as observed for EGF (37a).

The absence of increased levels of ETA receptor in the cells transfected with E6-E7 sequences alone suggests that other early genes or a cooperation among different viral genes could be involved in the upregulation of the ETA receptor. The biological significance of the overexpression of the ETA recep-
tor resides in the demonstrated capacity to enhance the autocrine mitogenic signal induced by endogenous ET-1. Both cell lines immortalized by the complete genome of HPV16 can grow in the absence of any growth factor added to the medium and in the presence of different levels of released endogenous ET-1. Zyzak et al. (49) reported the growth of HPV16-immortalized keratinocytes in the absence of any growth factor and postioned determination of the reason for the loss of requirement for bovine pituitary extract until the identification of other growth factors that are important for keratinocyte proliferation. Our results suggest that ET-1 is one of the essential growth factors involved in the growth control of HPV16-immortalized keratinocytes, as demonstrated by the dramatic decrease of cell proliferation in the presence of a specific ET_A receptor antagonist, BO123. The pathophysiologic role of the ET-1 autocrine loop in keratinocyte proliferation is open to speculation. As postulated for the EGFR, the increase of ET-1 receptors enhances the proliferation rate of HPV-infected cells in the presence of a normal amount of growth factor in the microenvironment and therefore could give a selective advantage to the infected cells. We speculate that in this immortalized population, a second event could account for the generation of tumor cells, or that ET-1 is directly involved in cell transformation. Increased levels of growth factor receptors are a common characteristic of human cancer cells and could likely play an important role in the maintenance of autonomous growth in cancer cells in vitro and in vivo. Furthermore, ET-1 is often expressed at higher levels in malignant tissue than in normal epithelia, suggesting that altered regulation of this growth factor and its receptor may contribute to cancer development (24).

Taken together, our data indicate a new mechanism in the growth control of HPV-immortalized keratinocytes mediated by the ET-1 autocrine loop. This conclusion is based on several observations: (i) ET-1 is released from HPV-transfected keratinocytes; (ii) all HPV16-transfected cell lines exhibit increased numbers of ET_A receptors compared to parental cells; (iii) ET-1 enhances \(^{3}H\)thymidine incorporation and cell proliferation in these cell lines; (iv) HPV-immortalized keratinocytes overexpress of a cDNA encoding an endothelin receptor. Nature (London) 382:817–820.

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**REFERENCES**


38. Venuti, A. Personal communication.