TGFβ-1 and TNFα Expression in the Epidermis of Patients with Epidermodysplasia Verruciformis

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In epidermodysplasia verruciformis (EV), the infection with specific human papillomaviruses (HPV) might be under control of the local immunosurveillance mechanisms related to cytokines produced by epidermal cells. We have investigated by in situ hybridization the expression of mRNA coding for TGFβ-1 and TNFα in the skin of patients with EV (n = 4) as compared to the skin lesions of patients with other premalignant (actinic keratosis; n = 5) or malignant (squamous cell carcinoma; n = 4) skin lesions, and to the skin of healthy individuals (n = 5). The expression of TGFβ-1 and TNFα mRNA was higher in the epidermis of EV patients as compared to the control skin from healthy individuals. The increased expression of mRNA for both cytokines was confirmed by northern blot analysis of RNA isolated from the skin lesions of the patient with EV. No specific signals for TGFβ-1 and TNFα were detected in actinic keratosis, and in cases of squamous cell carcinomas only single neoplastic cells were positive for TGFβ-1. It is conceivable that in EV TGFβ-1 and TNFα can be involved in the regulation of the growth and differentiation of HPV-infected keratinocytes and in the persistence of HPV-induced skin lesions. J Invest Dermatol 97:862–867, 1991

Epidermodysplasia verruciformis (EV) is a rare skin disorder characterized by massive infection with a variety of disease-specific human papillomaviruses (HPV) [1,2]. EV may be considered as a natural model of HPV-associated carcinogenesis in humans [3]. Both sporadic and familial cases of EV have been described, and an autosomal recessive inheritance has been suggested [4].

The clinical onset of EV is usually in childhood, and in about one-third [3,4] to one-half [2] of the cases, skin malignancies appear in adult life. The skin cancers that develop in EV patients are in situ, microinvasive, or invasive squamous cell carcinomas, usually with pronounced bowenoid atypia. They are usually multiple and appear mainly in the sun-exposed areas [1,5]. Carcinomas in EV patients may be locally destructive but very rarely metastasize [5].

Virologic studies (molecular hybridization and restriction enzyme analysis) have shown that the skin lesions in EV patients are associated with about 20 different HPV types, mainly EV-specific HPVs [2]. The detection of the HPV type is of great clinical importance because the genomes of HPV 5 and HPV 8 and less frequently HPV 14 and HPV 17 were found in both primary carcinomas of EV patients [1,2,5] and in some metastatic tumors [2,6].

The mechanisms responsible for infection with EV-specific HPV that are harmless for the general population and the factors involved in malignant conversion are not known. There is increasing evidence that HPV infection may be under the control of the host immune system [7]. This is strongly supported by the detection of EV-specific HPV in the skin lesions and tumors of heavily immunosuppressed patients [8–11], and by a derangement of cell-mediated immunity (CMI) found in the majority of EV patients [12–15], including a specific defect of natural cell-mediated cytotoxicity [16] or T-cell response [17] to the keratinocytes infected with these HPV.

In spite of massive infection with potentially oncogenic HPV, the tumors develop only in single lesions and, by absence of adverse factors, are not invasive. We presume that some local immunosurveillance mechanisms must be involved in the control of this infection and the development of cutaneous malignancies.

The present study was aimed at assessing the expression of two immunomodulatory cytokines, i.e., TGFβ-1 and TNFα [18], in the skin lesions of patients with EV, as compared to patients with other premalignant (actinic keratosis) or malignant (squamous cell carcinoma) skin lesions.

**PATIENTS AND METHODS**

**Patients** The clinical data and results of virologic studies in the patients with EV are presented in Table I. The control group included five patients with actinic keratoses (ages: 57–71 years), four patients with single squamous cell carcinomas (ages: 58–86 years), and five healthy volunteers (ages: 39–54 years). All biopsies were performed under local anaesthetic with the consent of the patients. The diagnoses have been confirmed by routine histologic examinations.

**Preparation of Tissue Sections** Biopsies were immediately frozen in liquid nitrogen and stored at −80°C. Frozen sections mea-
suring 5 μm were mounted onto slides, air dried, fixed in 4% paraformaldehyde in PBS for 1 min, and finally kept in 70% ethanol at 4°C.

**Preparation of RNA Probes** RNA probes were prepared as described previously [19]. Briefly, a 780-bp fragment of the TGFβ-1 clone [20] (kindly provided by Dr. R. Derynck, San Francisco, CA) and a 820-bp fragment of the TNFα clone [21] (kindly provided by Genentech, San Francisco, CA) were subcloned into the Eco RI BamH I site of the Gemini 3 vector (Promega Biotec, Madison, WI) and the Eco R1 site, respectively. Following linearization with an appropriate restriction enzyme, in vitro transcription was carried out for 60 min at 37°C in the presence of 1 μg DNA template, 15 mM dithiothreitol (DTT) (Sigma, St. Louis, MO), 20 U RNA-in (Promega Biotec), 25 μmol each ATP, GTP, CTP (Boehringer, Mannheim, Germany), and 12.5 μmol cold UTP, 50 μCi α-35S-UTP (specific activity 1268 mmol; Amersham-Buchler, Buckinghamshire, U.K.), and 1 U of SP6 or T7 polymerase (Promega Biotec), depending on whether sense or anti-sense probe was synthesized. After digestion of the DNA templates with 1 U of DNase I (Promega Biotec), RNA probes were precipitated with ethanol and 3 M NaAc.

**In Situ Hybridization** In situ hybridization was carried out as described elsewhere [19]. Briefly, frozen sections were washed twice in 2×SSC followed by an acetylation step in acetic anhydride-0.1 M triethanolamine pH 8.0 (Merck, Darmstadt, Germany). The slides were then rinsed in 2×SSC (1×SSC = 0.15 mM sodium chloride, 15 mM sodium citrate), incubated in 0.1 M Tris-HCL pH 7.0 (Boehringer), 0.1 M glycine (Merck, Darmstadt, Germany) for 30 min washed again in 2×SSC, and subsequently equilibrated in 50% formamide (Fluka, Buchs, Switzerland), 2×SSC at 50°C. Hybridization was performed at 50°C for 3 h by using 1×106 cpm/section. After hybridization, slides were rinsed in 50% formamide, 2×SSC for 20 min at 52°C followed by three washes in 2×SSC. After treatment of the section with RNase A (100 μg/ml, Sigma) and RNase T1 (1 μg/ml, Boehringer) in 2×SSC at 37°C for 30 min, slides were washed in 50% formamide, 2×SSC at 52°C for 5 min, then rinsed in 2×SSC and finally dehydrated in ethanol. Autoradiography was performed using NTB2 nuclear track emulsion (Eastman Kodak, Rochester, NY).

After exposure of the slides for 4–5 d at 4°C, slides were developed (D19, Kodak, Stuttgart, Germany), fixed (AL4, Kodak), and finally stained with hematoxylin-eosin. In order to ensure the integrity of tissue RNA and the specificity of the hybridization, the following controls were performed: 1) a human suprabasal keratin probe [22] and 2) sense RNA probes of TGFβ-1 and TNFα.

**Northern Blot Analysis** Frozen biopsies were cut on dry ice and homogenized in 4 M guanidine-isothiocyanate containing 0.1 M 2m-mercaptoethanol. After centrifugation with 3000 × g at 4°C for 15 min, supernatants were extracted with phenol and chloroform. RNA was then separated by ultracentrifugation over a cesium chloride cushion according to published protocols [23].

For northern blot analysis, 10 μg total RNA were separated by gel electrophoresis in 1% agarose under denaturing conditions and then blotted onto a nylon membrane (Genescreen, Dupont, USA). As a control for the amount and integrity of RNA blotted, the membrane was stained with methylene blue 0.04% in 0.2 M NaAc (pH 5.2). After crosslinking with UV-light, filters were prehybridized at 42°C in 50% formamide, 50 mM sodium phosphate, 5×SSC, 5× Denhardt’s solution, and 250 μg/ml herring sperm DNA for 3 h. Hybridization with the cDNA probes was carried out with 32P-labeled nick-translated cDNA probes for 12 h in the same solvent.

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**Table I.** Clinical Data of Patients with Epidermodysplasia Verruciformis and HPV Types Detected in Their Lesions

<table>
<thead>
<tr>
<th>Patient Number</th>
<th>Age/Sex</th>
<th>Age at Onset of EV</th>
<th>Age at First Cutaneous Malignancy</th>
<th>HPV Type*</th>
<th>Cutaneous Malignancy†</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>47/F</td>
<td>5</td>
<td>17</td>
<td>5, 8, 9, 14, 17, 19, 20, 22, 24, 36, and 3</td>
<td>++</td>
</tr>
<tr>
<td>2</td>
<td>53/M</td>
<td>6</td>
<td>37</td>
<td>5, 36, and 3</td>
<td>++</td>
</tr>
<tr>
<td>3</td>
<td>37/F</td>
<td>4</td>
<td>22</td>
<td>5, 36, and 3</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>55/F</td>
<td>11</td>
<td>51</td>
<td>2 and 5, 9, 17, 19, 22</td>
<td>±</td>
</tr>
</tbody>
</table>

* The prevalent HPV type is underlined.
† ±, actinic keratoses with features of Bowen's atypia or single carcinomas in situ in the past. +, in situ or microinvasive carcinomas. ++, multiple carcinomas, invasive at some sites.

**Figure 1.** In situ hybridization of normal human skin with anti-sense RNA probes for TGFβ-1 (a) and TNFα (b) revealed single silver grains in some basal and suprabasal keratinocytes. Magnification × 664.
Figure 2. In situ hybridization of tissue sections from EV lesions (a,c,e,f) and from uninvolved skin (b,d) of patient no. 1 for TGFβ-1 (a,b) and TNFα (c,d) mRNA. Control experiments using a sense RNA probe for TGFβ-1 (e) revealed, apart from single background silver grains, no specific hybridization. Similar results were obtained for TNFα sense RNA probe. Hybridization of tissue sections with anti-sense RNA probe for keratin K10 showed distinct labeling of the upper suprabasal keratinocytes (f). Magnification ×664.
Figure 3. In situ hybridization of tissue sections from the premalignant skin lesions of EV patient 2 with anti-sense RNA probes for TGFβ-1 (a) and TNFα (b) revealed also a strong signal for these cytokines. Magnification X 664.

After hybridization, filters were washed twice in 2 × SSC, 0.1% SDS at room temperature for 15 min followed by two washings in 0.1 × SSC, 0.1% SDS at 55°C for 15 min. Filters were then exposed at −80°C to a radiosensitive film (Kodak, X-Omat AR). Before rehybridization, filters were stripped in 0.1 × SSC, 1% SDS for 20 min at 100°C.

RESULTS

Localization of TGFβ-1 and TNFα in the Skin of Patients with Epidermodysplasia Verruciformis

When TGFβ-1 and TNFα anti-sense RNA probes were applied onto the sections of normal human skin, only weak reaction was detected within some basal and suprabasal keratinocytes of the epidermis (Fig 1). Hybridization of mRNA with anti-sense RNA probes on tissue sections from patients with EV revealed a strong signal in the epidermis (Figs 2 and 3; Table II). The expression of mRNA for both cytokines was higher in the biopsies from the skin lesions, as compared to the clinically uninvolved skin from the same anatomic areas (Fig 2b, d). The highest expression of mRNA was detected in the skin of patients with the most pronounced cutaneous malignancies (patients 1 and 2), and the lowest expression was detected in patient 4, without active pre-malignant or malignant lesions (Table II).

Table II. TGFβ-1 and TNFα mRNA Expression in the Skin of Patients with Epidermodysplasia Verruciformis, Detected by In Situ Hybridization.

<table>
<thead>
<tr>
<th>Patient Number</th>
<th>Localization of Biopsies</th>
<th>TGFβ-1</th>
<th>TNFα</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>Suprascapular area</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>2</td>
<td>Suprascapular area</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>3</td>
<td>Neck</td>
<td>++++</td>
<td>++</td>
</tr>
<tr>
<td>4</td>
<td>Neck</td>
<td>++</td>
<td>+</td>
</tr>
</tbody>
</table>

* +, Weak signal (single silver grains in the basal or suprabasal layer). +++, moderate signal (grains in the lower suprabasal layers). ++++, strong signal (grains throughout the squamous cell layer).

In order to check the specificity of hybridization of the RNA probes on skin sections, 35S-labeled TGFβ-1 and TNFα sense RNA probes were used as negative controls, and did not reveal any cellular signals (Fig 2c). The integrity of mRNA was demonstrated by applying a human suprabasal keratin probe on the tissue sections. The hybridization revealed distinct labeling of the epidermis, indicating the presence of undegraded message (Fig 2f).

In the tissue sections obtained from patients with actinic keratoses, no specific signal for TGFβ-1 or TNFα was detected and in cases of squamous cell carcinomas single neoplastic cells were weakly positive for TGFβ-1 mRNA (data not shown).

Northern Blot Analysis

In order to confirm the data obtained by in situ hybridization, we used a second independent procedure, i.e., northern blot analysis of tissue RNA. Determination of mRNA steady-state levels revealed specific signals for TGFβ-1 and TNFα with tissue RNA isolated from the skin lesions of EV patient 2, but not with RNA isolated from control skin (Fig 4). The 32P-labeled
cDNA probes revealed two mRNA species of 2.4 kb for TGF-β1 and of 2.2 kb for TNF-α as calculated by the 18S and 28S ribosomal RNA.

DISCUSSION

Using in situ hybridization, we found an increased expression of mRNA coding for TGFβ-1 and TNFα in the epidermis of skin lesions of patients with EV. The increase was confirmed by northern blot analysis of the tissue RNA. The highest expression of mRNA for TGFβ-1 and TNFα was found in EV patients with cutaneous malignancies. In contrast to that, no message for TGFβ-1 and TNFα could be detected in cases of actinic keratosis in non-EV patients, and only weak signals were found in the tissue sections of squamous cell carcinomas.

The implications of the increased expression of TNFα and TGFβ-1 in the lesional epidermis of EV patients are unknown, but this expression could be due to infection and/or transformation of keratinocytes with HPV. This is supported by our recent study showing that keratinocytes bearing HPV16 DNA copies (Skv cells) are capable of producing high amounts of TNFα and inhibitor of its activity.* TNF-α can act as a negative autocrine growth factor for these cells. Also, the second cytokine studied, TGFβ-1, is a widely distributed mediator inhibiting the growth of many epithelial cells [24].

Although TGFβ-1 is a potent inhibitor of the growth of normal epithelial cells, proliferation of transformed keratinocytes, squamous cell carcinomas, retinoblastoma cells, and other tumor cell types is not abrogated by this cytokine [24]. For some types of these cells (e.g., retinoblastoma), it has been demonstrated that lack of responsiveness to the inhibitory action of TGFβ is due to the absence of TGFβ receptors on the cell surface [25]. Finally, it could be speculated that unresponsiveness of EV keratinocytes to the inhibitory action of TGFβ may be due to the effect of some transforming proteins of EV-specific HPV with retinoblastoma gene product (pRB)-binding domains, as shown for HPV 16 [26].

The production of TGFβ-1 by EV keratinocytes could lead to the depression of local immunosurveillance mechanisms, as this cytokine has various immunosuppressive properties, including the inhibition of T-cell activity, IL-1-dependent antigen presentation, and natural killer and lymphokine-activated killer cell cytotoxicity [24].

The characteristic feature in patients with EV is a very low potential of cancers to invasive growth and to metastasize, even in cases with pronounced malignancy [1–3,5], as well as an accelerated process of wound healing [5]. Both TNFα and TGFβ have a crucial role in the connective tissue remodeling and the process of metastasis formation [24,27]. TNFα has proinflammatory and collagenase-stimulating activities, whereas TGFβ induces several protease inhibitors and decreases synthesis and secretion of enzymes degrading the extracellular matrix. Therefore, suppression of collagenase activity and induction of extracellular matrix protein synthesis by TGFβ may contribute, at least in part, to the lack of metastasis formation in patients with EV. Moreover, TGFβ-dependent enhancement of tissue repair could be responsible for the accelerated wound healing in patients with EV.

REFERENCES


* Autocrine growth limitation of human papillomavirus type 16 (HPV16)-harboring tumor keratinocytes by constitutively released TNFα: a possible surveillance mechanism against HPV-induced neoplasia. (Majewczyk et al, submitted)


