Tumorigenicity and Metastatic Behavior in Nude Mice of Two Human Squamous Cell Carcinoma Lines that Differ in Production of the Cytokine ETAF/IL-1

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The purpose of these studies was to determine whether the production of the cytokine epidermal cell thymocyte-activating factor (ETAf) by human squamous cell carcinoma (SCC) cells correlated with their tumorigenicity and metastatic potential in athymic nude mice. Cells of the human SCC line A431 produced rapidly growing subcutaneous tumors, few experimental lung metastases, and low levels of ETAf activity in vitro. In contrast, cells of the SCC Colo-16 line produced slower growing subcutaneous tumors, high numbers of experimental lung metastases, and a high level of ETAf activity in culture supernatants. The apparent relationship between production of ETAf and experimental metastasis formation was not consistent. Clonal populations of the SCC A431 and Colo-16 were isolated in vitro. The clones of Colo-16 varied in their ability to produce experimental metastases and in production of ETAf in vitro. However, the levels of ETAf production did not correlate with the propensity of the SCC cells to produce experimental metastases. We conclude that while the growth and metastasis of human SCC in nude mice may benefit from production of the cytokine ETAf, the ETAf production per se is not invariably linked with the capability of the SCC cells to metastasize. J Invest Dermatol 91:258–262, 1988

Normal and neoplastic keratinocytes produce a cytokine, termed epidermal cell derived thymocyte-activating factor (ETAf), that shares many of the various biologic activities of interleukin-1 (IL-1) [1–3]. Because ETAf can modulate a number of host immune and inflammatory responses, its production by epidermal cells may regulate local immune reactivity in the skin [3,4]. Some human squamous cell carcinomas (SCC) can produce ETAf [3]. Whether this property is related to other biologic properties of the tumor cells, such as the local growth and metastasis of the human tumor cells after implantation in athymic nude mice, is not known. Because the nude mouse lacks mature T-lymphocytes by definition, possible actions of the cytokine ETAf on host cells other than T-lymphocytes can be studied. For example, in vitro studies have shown that ETAf is a chemoattractant for neutrophils and an autocrine growth factor for keratinocytes [3,5]. Also, recent studies have shown that ETAf is a chemoattractant for tumor cells [6]. IL-1 has been shown to stimulate a variety of responses in nonlymphoid cells, including endothelial cells, fibroblasts, and astroglial cells [7–9]. As interactions between tumor cells and normal cells, by cell contact or via diffusible mediators, are integral steps in the process of tumor invasion and metastasis [10,11], it is pertinent to ask whether a tumor cell-derived factor with properties similar to those of IL-1 can be involved in these processes. In this study, we examined two human SCC, the A431 and Colo-16 cell lines which differ in the level of production of ETAf/IL-1. We investigated how this cytokine may be involved in tumorigenesis and metastasis of human SCC in athymic nude mice. Specifically, we asked the following questions: I) Does the production of ETAf influence the tumorigenic potential of human SCC in nude mice? II) Is ETAf production related to the ability of human SCC cells to metastasize in nude mice?

Methods and Materials

Cell Culture The two human SCC lines, Colo-16 [12] and A431 [13], were maintained in monolayer culture in Eagle's MEM supplemented with 10% FBS sodium pyruvate, L-glutamine (2 mM), non-essential amino acids, and 2x vitamin solution (Gibco, Grand Island, NY). The cultures were incubated at 37°C in a humidified atmosphere of 5% CO2–95% air. Both lines were examined for, and found free of, Mycoplasma, reovirus type 3, pneumonia virus of mice, mouse adenovirus, murine hepatitis virus, lymphocytic choriomeningitis virus, eclotremia virus, k-virus, Theiler's virus, Sendai virus, and lactate dehydrogenase virus (assayed by Microbiological Associates, Walkersville, MD). The human origin of the cells was verified by karyotypic analysis.
In Vitro Growth Rates
To determine growth rate in culture, 10^6 viable tumor cells were seeded in 60 mm diameter tissue culture dishes containing 6 ml of culture medium and incubated. At five daily intervals, cells from three dishes were harvested with 0.25% trypsin-0.12% EDTA and counted using hemocytometer. The mean cell number recovered per day was plotted against time, and the doubling time of the tumor cells in logarithmic growth was determined.

Animals
Athymic nude mice (NCr-nu/nu), 6 to 8 weeks old, were obtained from the Animal Production Area of the NCI-Frederick Cancer Research Facility. Mice were age-matched for each experiment. Throughout the experiments, the mice were maintained in laminar flow cabinets under specific pathogen-free conditions.

Tumor Cell Inoculation
Tumor cells were harvested from cultures in log-phase growth by washing the monolayers with Ca^{2+} and Mg^{2+} free Hank’s balanced salt solution (HBSS) and then briefly incubating them with a 0.25% trypsin-0.12% EDTA solution. The flask was tapped to dislodge the cells that were then resuspended in supplemented MEM (see above) and washed by centrifugation. The pellet was resuspended in HBSS, and the number of viable cells was determined by staining with a small aliquot with 0.2% trypan blue saline solution and examining the sample in a hemocytometer. The dye is excluded by viable cells.

Cells were resuspended in HBSS at appropriate concentrations for inoculation into mice. Suspensions of both lines were injected s.c. (lateral thorax) and i.v. (lateral tail vein) into groups of six mice each, at an inoculum dose of 10^5 tumor cells in 0.2 ml HBSS. The mice injected s.c. were examined twice weekly for tumor growth, and growth rates were determined from measurements of two perpendicular diameters. When tumors reached a mean diameter of 1.5 cm, the mice were anesthetized by Metofane and potassium chloride was used for anesthetizing the animals. The incision was performed and closed by suturing. The mice were killed 6 weeks after tumor excision and examined at necropsy for metastases. Tissues were fixed in 10% buffered formalin for histologic examination. Mice injected i.v. were killed 5 weeks after injection and examined for experimental metastases. Organs were fixed in 10% buffered formalin, and tumor nodules were counted by examining the lungs through a dissecting microscope.

In Vitro Cloning
In the next set of experiments we investigated whether the growth rate at a s.c. site and the propensity to produce experimental lung metastases subsequent to i.v. injection were related to the levels of ETAF production by the tumor cells. To do so, we isolated several clones from the A431 and Colo-16 cell lines. Tumor cells were harvested with trypsin-EDTA solution 24 h after fresh culture medium had been added to the culture flasks. The conditioned medium was saved for each cell line, centrifuged, and filtered through a 0.2 μm pore filter. The cell suspensions were washed by centrifugation to remove residual trypsin and suspended in 50% fresh/50% conditioned medium, at a concentration of 2.5 viable cells per milliliter. Aliquots of 0.2 ml were plated in each well of 96-well flat-bottomed plates, thus plating an estimated 0.5 cell/well. After 16 h incubation, wells with only one cell were marked; other wells were excluded from further study. Culture medium was replaced at 4–6 d intervals. When the cells became confluent, the cells were harvested with trypsin-EDTA and replated into 25 cm² tissue culture flasks. The clonal populations were expanded in culture, and serially passaged into larger tissue culture flasks.

ETAf Activity in Culture Supernatants
Two days after the addition of fresh culture medium, conditioned medium was collected from wells containing individual clones of A431 and Colo-16 at 75% confluency. The supernatants were tested for IL-1 activity in a mouse thymocyte proliferation assay as described in detail previously [14].

Tumorigenicity and Metastatic Ability of the Colo-16 Clones
Seven clones of Colo-16 were selected for further experiments, three with high ETAF activity and four exhibiting low ETAf activity, as assessed by the mouse thymocyte proliferation assay. Cells were injected s.c. at four different doses (5 X 10^5, 10^6, 5 X 10^6, and 10^7 viable cells per mouse). The i.v. inoculum was 10^6 cells per mouse in 0.2 ml HBSS.

To test whether the metastatic behavior of the tumor cells was affected by host natural killer (NK) cell levels, groups of mice were pretreated with anti-asialo GM-1 [15] (Wako Chemicals, Dallas, TX), diluted 1:20 (vol/vol) with sterile saline. Mice were injected i.p. with the antiseraum 6 d and 1 d before i.v. tumor cell injection. Control animals were injected with saline only.

The mice injected s.c. were examined three times per week for the appearance of palpable tumors, and measurements of the tumors were then taken twice weekly. The mice injected i.v. were killed 6 weeks after injection and necropsied. Tumor deposits in the lungs and in other organs were counted, and sample tissues were fixed in 10% buffered formalin.

Histology
Formalin-fixed tissues were embedded in paraffin, and 5 μm thick sections were stained with hematoxylin and eosin.

In Vitro Effects of ETAF/IL-1
The in vitro plating efficiency and growth of Colo-16 and A431 cells in ETAf/IL-1 conditioned media was assessed. Colo-16 and A431 cells were cultured at different cell densities (10^5 to 5 X 10^6 cells per well) in 96 well microwell plates. Conditioned media from confluent cultures of A431 and Colo-16 cells were centrifuged and filtered. Aliquots were heated at 80°C for 10 min to inactivate the cytokine activity [3]. The conditioned media was mixed with the culture medium in proportions of 25%, 50%, or 75%. Relative cell numbers in the plates were assessed at 24 h, 3 d, and 5 d, using the hydroethidine (HET) incorporation method [16,17]. The cells were incubated with 28 μg/ml HET in 0.1 ml PBS for 30 mins, washed with PBS, then lysed by the addition of 0.1% Triton-X100. The fluorescent emission of HET was measured using a Microfluor reader (Microfluor, Dynatech, Alexandria, VA), and the relative fluorescence units in the test wells compared with control wells, i.e., the cells plated in non-conditioned culture medium.

The effects of the conditioned media on human fibroblasts (SRB-30F), isolated from a surgical specimen of squamous cell carcinoma, and A375 human melanoma cells were also evaluated. 2.5 X 10^3 cells were plated per well in 96-well microtiter plates. Conditioned medium from Colo-16 cultures was added in different concentrations and the relative cell number per well assessed after 3 and 5 d culture, using the HET method described above.

Results
Tumorigenicity of A431 and Colo-16 SCC
Following the s.c. injection of 10^6 viable cells in the lateral thoracic region of nude mice, both tumor cell lines produced progressively growing tumors. The appearance and growth rate of the A431 tumors was more rapid than the Colo-16 tumors (Fig 1, Table 1). No macroscopic metastases were found in any of the mice killed 6 weeks after the excision of the s.c. tumors, and histologic examination of the lungs of these mice did not reveal microscopic metastases.

Histology
The histology of the A431 tumors growing s.c. was that of a poorly differentiated squamous cell carcinoma, with occasional foci of keratin (Fig 1). The histology of the Colo-16 tumors was characteristic of a well-differentiated SCC, with numerous keratin pearls (Fig 2). Neutrophil infiltration was apparent only in areas of central necrosis in tumors of both SCC lines.

Experimental Metastasis Formation by A431 and Colo-16 Cells
Intravenous injection of 10^6 viable A431 cells into nude mice produced few lung tumor colonies (median = 1, Table 1). Histologic examination of lungs sectioned at several levels did not reveal additional, microscopic tumors. In contrast, the i.v. injection of 10^6 Colo-16 cells produced macroscopic experimental metastases in all the recipient mice (median = 63, Table 1). Occasional extra-pulmonary metastases, predominantly in lymph nodes and muscle, were observed in mice injected with cells from either of the SCC lines.
**Figure 1.** Histologic section of A431 SCC growing s.c. in a nude mouse. Hematoxylin and eosin (X 140).

**Figure 2.** Histologic section of Colo-16 SCC growing s.c. in a nude mouse. Hematoxylin and eosin (X 140).

**Figure 3.** ETF/IL-1 activity in culture supernatants from A431 and Colo-16 SCC cells. The relative activity of each supernatant fluid is expressed as a percentage of the activity produced by a 1:10 dilution of a positive IL-1 control sample (39,307 cpm ³H-thymidine incorporated). Activity in the control supernatant was reproducibly less than 5% of the positive control shown in this figure.

**ETAF—Activity in Culture Supernatants of A431 and Colo-16 SCC Cells**  Culture supernatants of A431 and Colo-16 clonal populations were assayed for ETF/IL-1 activity in a mouse thymocyte proliferation assay as described by Lachman et al. [14]. In repeated assays, the culture supernatants collected from Colo-16 cells contained greater ETF/IL-1 activity than the culture supernatants collected from A431 cells. The relative ETF/IL-1 activities in supernatants from A431 parental line cells and eight clones and Colo-16 parental line cells and 14 clones are shown in Fig 3. The activity of each supernatant fluid is expressed as a percentage of the activity produced by a 1:10 dilution of a positive IL-1 control sample (from human leukemic monocytes [14]). The A431 parent line cells and the clonal derivatives produced little ETF/IL-1 activity, whereas those of the Colo-16 produced widely ranging levels of IL-1 activity. Three of the Colo-16 clonal populations produced higher levels of activity than the parental line (clones 4, 15, and 17), while the other clonal lines examined produced lower activity than the parental line. Seven clones were selected for further comparison with the Colo-16 parental line; these were the three clones with higher ETF/IL-1 activity and the four clones with lower activity in the assay (clones 5, 7, 8, and 14).

**Comparison of the Release of ETF and the In Vivo Behavior of Colo-16 Parental Line and Clones**  The in vitro growth rates of the parental Colo-16 line and the seven selected clones were calculated in order to determine whether expression of high levels of ETF was related to a faster (or slower) growth rate. With the exception of clone-17, which had a much longer doubling time than the other clones and the parent line, all the variants tested exhibited similar in vitro growth rates (Table II). Thus, we found no correlation between the level of ETF synthesis and cell doubling time in vitro.

The tumorigenic abilities of the seven clones of Colo-16 were assessed by s.c. injection of cells at four different cell doses. No tumors were produced by the injection of 5 X 10⁴ cells of any clonal population or of the parental cell line. The production of progressively growing tumors subsequent to s.c. injection of 10⁵, 5 X 10⁵, and 10⁶ cells is shown in Table II. The latent period for tumor formation was calculated from the mean time before tumors arising from the s.c. injection of 10⁶ cells measured 3 mm in diameter (results from groups of six mice). All mice (6/6) injected with 10⁶ cells of the parental Colo-16 line, and of clones 4, 7, 8, and 13 developed s.c. tumors, with a latent period of 13 to 19 d. However,

| Table 1. Tumorigenicity and Production of Experimental Metastasis by A431 and Colo-16 SCC Injected into Nude Mice |
|-----------------|----------------|----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| SCC             | In Vitro        | Tumor          | Experimental    |
|                 | doubling        | Take           | metastasis      |
|                 | Time a          | Latency b      | Incidence       | Median (range)  |
| Colo-16         | 18.4            | 10/10          | 13.9 (± 6)      | 11/11           | 63 (38 - 150)   |
| A431            | 20.6            | 10/10          | <5              | 10/13           | 1 (0 - 12)      |

a In hours; calculated over 120 h, while the cells were in logarithmic growth.
b 10⁶ cells injected s.c.
c Mean interval in days before detection of a 3-mm diameter s.c. tumor resulting from injection of 10⁶ tumor cells.
d Experimental metastasis 5 weeks after i.v. injection of 10⁶ tumor cells. Incidence, number of mice with experimental metastases/number of mice injected, and median number (and range) of lung tumor colonies.
### Table II. Biologic Properties of Clones of Colo-16 SCC

<table>
<thead>
<tr>
<th>In Vitro</th>
<th>Tumorigenicity s.c. c</th>
<th>Cell dose</th>
<th>In Vivo</th>
<th>Experimental metastases d</th>
<th>Anti-asialo GM-1 pretreatment c</th>
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<td>Latency (day) a</td>
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<td>Median (range)</td>
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<td>3(0-18)</td>
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</table>

a In hours; calculated over 120 h, while cells were in logarithmic growth phase.
b Relative levels of ETAF activity in culture supernatant. (See “Materials and Methods”).
c Number of s.c. tumors/number of mice injected at each dose.
d Mean interval in days before a 3-mm diameter s.c. tumor resulting from the injection of 10^6 cells was detected.
e Experimental metastasis 6 weeks after the i.v. injection of 10^6 tumor cells. Incidence; the number of mice with experimental metastasis/number of mice injected; and median number (and range) of lung tumor colonies.

The experimental metastatic potential of the Colo-16 clones was assessed by i.v. inoculation of 10^6 viable tumor cells into nude mice and counting lung nodules 6 weeks later. Inoculation of the parent line cells produced numerous lung nodules (median = 80). While all 7 clones did form lung nodules in at least one recipient mouse, none of these cell lines produced as many lung colonies as the parental line, and four clones produced very few lung tumor colonies (clones 4, 17, 5, and 7). Production of lung tumor colonies following i.v. inoculation did not appear to be related to production of ETAF activity. We base this conclusion on the data demonstrating that tumors cells that produced high levels of ETAF in vitro (clones 17 and 4) and those that produced little ETAF activity (clones 5 and 7) were all weakly metastatic compared with the parental Colo-16 tumor line (Table II).

To test whether the low- and high-ETAF-producing isolates differed in their sensitivity to NK-cell activity in nude mice, the cells were injected into mice pretreated with anti-asialo GM-1 and into control mice (saline injected.) Reduction of NK cell levels by this pretreatment had no effect on the experimental metastatic potential of the human tumor cells.

Collectively, although the seven clones of Colo-16 tested in vivo differed in production of s.c. tumors, tumor growth rates, and experimental metastatic potential, these differences did not correlate with the in vitro production of ETAF.

### In Vitro Assays of ETAF/IL-1 Activity in SCC Conditioned Media

In repeated assays for the plating efficiency and in vitro growth of the SCC cells, assessed by the incorporation of a vital fluorescent dye, HET, no significant stimulatory effect of conditioned culture medium was seen (Fig 4). Conditioned media from A431 or Colo-16 cells did not significantly alter the growth of either of the SCC cells compared with non-conditioned medium, or heat inactivated conditioned media. Parallel assays for ETAF/IL-1 activity in the conditioned media showed that there were significant levels present. In one set of experiments in which Colo-16 cells were plated with a polyclonal antibody to IL-1 (1:10 dilution) the plating efficiency and growth of the SCC cells over 5 d was not altered (data not shown).

In contrast the same supernatants did modulate the in vitro growth of the human melanoma cell line A375, and also human fibroblasts (Fig 4). The Colo-16 conditioned media inhibited the proliferation of A375 cells (80% inhibition, compared with A375 cells cultured in fresh medium or self-conditioned medium for 5 d). The fibroblasts showed a greater than twofold increase in cell number in the presence of the Colo-16 conditioned medium (180 to 240% of control values) (Fig 4). A431 conditioned medium was less effective in modulating the growth of the A375 and SRB-30F cells.

Figure 4. In vitro effects of Colo-16 supernatant on cell growth. Each bar represents the relative cell number, assessed from the incorporation of the vital fluorescent dye HET, of four different cell lines cultured in Colo-16 conditioned medium for 5 d. The initial plating density was 2 x 10^4 cells per well of a 96-well microwell plate. The results shown are the mean and s.d. of triplicate wells of a representative assay. C: control, 100% fresh culture medium; 3:1-75% fresh medium, 25% conditioned medium; 1:3 = 25% fresh, 75% conditioned medium. Y axis units are RFU, relative fluorescent units (see Refs 16 and 17).
DISCUSSION

Polypeptide growth factors can modulate the proliferation of tumor cells in vitro and in vivo [18]. Some factors are produced by tumor cells, acting as autocrine growth stimulators [19] or stimulating normal cells, e.g., tumor angiogenesis factor acting on capillary endothelial cells [11]. The production of diffusible mediators may therefore influence the ability of tumor cells to grow in different organ sites in vivo. The present report concerns two human SCC cell lines that differ in production of ETAF, a cytokine with multiple biologic activities [1–3]. We show that both tumor cell lines were tumorigenic in the s.c. site of nude mice, but that the Colo-16 cell line, with high ETAF activity had a greater propensity for seeding colonies in distant organs (principally lungs) after i.v. inoculation compared with A431 cells, which produced low levels of ETAF activity. However, A431 cells injected s.c. produced rapidly growing tumors sooner than the same number of Colo-16 cells. The comparatively increased ability of Colo-16 cells to produce experimental metastases is, therefore, not a consequence of a more rapid growth rate, but presumably is related to other cellular properties.

Clonal analyses of animal and human tumors have shown that neoplasms are composed of numerous subpopulations of cells with distinctly different biologic properties, such as morphology, growth rate, antigenicity and immunogenicity, hormone receptors, drug-sensitivity, and potential for invasion and metastasis [10]. In order to test whether ETAF production by human SCC cells influenced tumor growth and metastasis in nude mice, we isolated clonal populations from the high-ETAF producing Colo-16 cell line. The culture supernatants of the clones were tested for ETAF activity, and the different populations were found to be heterogeneous for this phenotype. The seven clonally derived populations that were studied also varied among each other and from the parent tumor line in their experimental metastatic potential and growth rates in vivo. The differences in biologic behavior of the clonal populations following injection into nude mice were not, however, related to the levels of ETAF activity detected in the respective culture supernatants. For this reason, the difference in metastatic behavior recorded for the SCC A431 and SCC Colo-16 cannot be simply related to the fact that the A431 cells produce low levels of ETAF activity, and the Colo-16 cells produce high levels.

ETA F shares several properties with IL-1 [3], and thus may play an important role in the immune response in the skin. In addition to its role in the activation of T cells, IL-1 acts as a growth factor for non-lymphoid cells such as fibroblasts [7] and astrocytes [8]. IL-1 has also been reported to have direct cytototoxic activity against a human melanoma cell line [20]. Indeed, culture supernatants from Colo-16 and A431 cultures, shown to have ETAF activity by the IL-1 thymocyte assay [14], were found to inhibit growth of the same human melanoma cell in vitro and to stimulate skin fibroblast proliferation. The in vitro growth assays using SCC conditioned medium did not indicate that exogenous ETAF/IL-1 either stimulates or inhibits the growth of the SCC cells, although for normal keratinocytes ETAF is an autocrine factor [5]. Production of the cytokine ETAF by neoplastic cells may have profound effects in the local tissue environment; for example, extracts of human basal cell carcinomas were found to express IL-1 activity, and concomitantly to stimulate production of skin fibroblast collagenase in vitro [21]. In addition, ETAF has been found to be chemotactic for certain tumor cells and to increase tumor cell adhesion to vascular endothelium (Sauder and Orr, unpublished data), although, again, it does not stimulate proliferation in vitro [6]. Although in our present study the production of ETAF by two established cell lines of human SCC did not correlate with the ability of these cells to proliferate in nude mice, the role of ETAF in the biology of skin cancers requires further study. Because the Colo-16 and A431 cell lines originated from SCC of different anatomical sites (skin and vulva, respectively [12,13]), we are now investigating the ETAF production by freshly isolated human SCC removed from different sites. This property will then be compared with the biologic behavior of the tumors subsequent to implantation into athymic nude mice.

REFERENCES