ABSTRACT
Eosinophilia in tissues and/or circulating blood is known to be associated with a wide variety of malignancies but the role of the eosinophil in neoplastic conditions is not known. Using the cheek pouch of the Syrian hamster as an experimental model for oral carcinogenesis, it has recently been shown that eosinophils at sites of developing oral cancer express the multifunctional cytokine, transforming growth factor α (TGF-α). This study investigated the time course of eosinophil infiltration, tissue eosinophilia associated with malignant epithelium, and eosinophil-derived TGF-α mRNA during the 16-week 7,12-dimethylbenz[a]anthracene (DMBA)-induced oral cancer development process. The results reveal that the occasional eosinophil is normally present in the lamina propria of hamster oral mucosa. With progressive DMBA treatments, there is an increase of eosinophils infiltrating into the lamina propria. By weeks 12–16, the number of eosinophils is significantly higher in DMBA-treated pouches than in control pouches treated with the vehicle mineral oil alone. Analysis of the infiltrating eosinophils into fully developed hamster oral carcinomas reveals that tissue eosinophilia is associated with 78% of the stromal areas associated with malignant epithelium, while only 7% of sites associated with non-tumor oral epithelium (normal, hyperplastic-dysplastic) exhibited eosinophilia. Furthermore, the majority of the eosinophils associated with malignant epithelium were found to contain TGF-α mRNA. The number of TGF-α mRNAs containing eosinophils associated with malignant oral epithelium is significantly higher than that associated with nonmalignant oral epithelium. Together, these results suggest that eosinophils are recruited to tumor-developing sites, that they predominantly associate with malignant epithelium, and that most tumor-associated eosinophils express the cytokine TGF-α.

INTRODUCTION
Tissue and blood eosinophilia is known to be associated with a number of neoplasms (1). Clinical correlative studies of patients with either blood or tissue eosinophilia, or both, have suggested that tissue eosinophilia at the tumor sites can be "protective," whereas blood eosinophilia may be an indicator of tumor metastasis (1). In order to define the possible roles of the eosinophil in the host-tumor development process, an animal tumor model is necessary for the study of this cell during the tumor development process as well as the cellular and molecular interactions at the tumor sites. The cheek pouch of the Syrian hamster is the most widely used model for experimental oral carcinogenesis (2). Periodic topical application of a specific carcinogenic chemical, such as DMBA, can lead to the consistent production of epidermoid carcinomas in a relatively short period of time (~14 weeks). Topical application of 0.5% DMBA in mineral oil, three times a week, induces hyperplastic changes in 4 weeks, dysplasia in 4–6 weeks, carcinoma in situ in 6–8 weeks, carcinoma in 8–10 weeks, and invasive carcinoma in 10–14 weeks. These histopathological alterations of the oral epithelium are similar, if not identical, to those in biopsies of human oral malignant and premalignant lesions.

Recently, our laboratory has shown that eosinophils infiltrated into DMBA-induced hamster oral cancers represent a major source of the multifunctional cytokine TGF-α (3). TGF-α production has since been confirmed in human eosinophils (4). Although eosinophilia has been described with a number of neoplastic conditions, including head and neck tumors, a thorough study of this cell in an animal cancer model has not been reported previously. The linkage of this cell with production of TGF-α, which has been implicated in the malignant transformation of epithelial tissues, further merits the careful delineation of this finding in an animal model. Such studies in a well-defined animal cancer model are critical to further understanding the role of this granulocytic leukocyte in malignant transformation and other pathological processes.

This paper represents an initial effort toward better understanding the participation of the eosinophil in the malignant transformation of oral mucosal tissue using the well-characterized hamster oral cancer model. We investigated the kinetics of eosinophil infiltration into the hamster buccal pouch during the DMBA-induced oral cancer development process, the association of tissue eosinophilia and transformed oral epithelium, and the production of TGF-α by the infiltrating eosinophils.

MATERIALS AND METHODS
Animals and Tissues. Thirty male Syrian golden hamsters, 60–90 days old, were purchased from the Charles River Breeding Laboratories, Wilmington, MA. Hamsters were housed 4/cage in an air-conditioned (24°C) animal room on a 12-h light-dark cycle and fed with a commercial stock diet (Purina Formula Chow) and tap water ad libitum.
Epidermoid carcinomas were induced in the cheek pouches of Syrian hamsters according to the protocol of Shklar (5). This is a 14-week tumor induction protocol with the DMBA-treated hamster oral epithelium developing defined histopathological lesions (hyperplasia, dysplasia, and carcinoma). The chemical carcinogen was a 0.5% solution of DMBA (0.5 g/100 ml; D-3254; Sigma Chemical Co., St. Louis, MO) dissolved in mineral oil (U.S.P.). The left buccal pouch of the hamsters was painted three times weekly with either 0.5% DMBA in mineral oil or mineral oil only, using a No. 4 soft sable brush. The body weight of the hamsters was recorded every week. Buccal pouches of hamsters were grossly examined at weekly intervals for epithelial changes and tumor development from week 2 to week 16. Four hamsters from both the DMBA treatment and mineral oil control groups were sacrificed at the end of weeks 0, 6, 8, 10, 12, 14, and 16 and both pouches were excised. The excised pouches were immediately fixed in freshly prepared 4% paraformaldehyde at 4°C for 2 h and then processed for routine histology.
Specific Staining for Eosinophils. Tissue eosinophils were identified by staining four contiguous sections with the following histochemical and peroxidase reagents: hematoxylin and eosin; Giemsa (3, 4); DAB-Ni (6); Giemsa and DAB-Ni. Sections stained with DAB were pretreated with 0.3% H₂O₂ for 10 min to block the endogenous per-
oxidase of neutrophils and RBC (6). Hamster eosinophilic peroxidase is resistant to such H2O2 blocking. Eosinophils are primarily identified by the combination of DAB and Giemsa staining. For the in situ hybridization studies, the tissue sections were counterstained with Giemsa (Fisher Scientific, Orangeburg, NY). Following in situ hybridization, rhodamine fluorescence is necessary to identify eosinophils, as described previously (3, 4).

Statistical Analysis of Data. The following statistical tests were used. Student's t test was used to determine the statistical significance of differences between mean numbers of infiltrated eosinophils at different times of DMBA treatment compared to the corresponding mineral oil controls (7). Student's t test was again used to test the significance in the differences in mean number of eosinophils labeled for TGF-α mRNA associated with malignant or nonmalignant oral epithelium. The Fisher exact probability test was used to assess the statistical significance of tissue eosinophilia associated with malignant versus non-tumor oral epithelium in tumor-bearing hamster cheek pouches (7).

In Situ Hybridization. DMBA and mineral oil-treated hamster cheek pouch tissues were processed for in situ hybridization and labeled for TGF-α mRNA as described previously (3). The molecular probes used were antisense and sense [35S]UTP-labeled riboprobes of the hamster TGF-α complementary DNA encoding for the mature peptide (8). Special precautions were taken to avoid nonspecific annealing of the riboprobes to the eosinophils, as described previously (3, 4).

RESULTS

Profile of Eosinophil Infiltration into the Cheek Pouch during DMBA-induced Oral Carcinogenesis. Fig. 1A demonstrates the tissue eosinophilia typically seen in a hamster cheek pouch that had been treated with DMBA for 14 weeks. Cytoplasm of tissue eosinophils is prominently stained by the DAB-Ni and appears dark brown, which permits easy identification of the eosinophil in the light blue Giemsa-counterstained background (Fig. 1). Four control (mineral oil-treated) and four DMBA-treated
Hamster cheek pouches from weeks 0, 6, 8, 10, 12, 14, and 16 were examined for infiltrated eosinophils by the DAB-Ni/Giemsa staining method. The results are quantified and summarized in Table 1.

Eosinophils are present in normal hamster cheek pouches in low but detectable numbers (1.5 ± 1.3 (SD)/10 ×200 fields). They are primarily located subepithelially, between a layer of skeletal muscle and the basement membrane of the oral epithelium. In both the DMBA- and mineral oil-treated groups, there is a progressive increase of infiltrating eosinophils as treatment proceeds. The magnitude of increase is higher for the DMBA-treated pouches (Table 1). Because of the experimental variability and the low number of animals sacrificed at each time point, data was pooled into the following three groups: week 0; weeks 6–10; and weeks 12–16. The number of eosinophils infiltrated into the DMBA pouches in the week 12–16 group is significantly higher than the mineral oil-treated pouches from the same time points (P < 0.001). This is illustrated in Fig. 2.

**Association of Eosinophilia with Malignant Hamster Oral Epithelium.** Our laboratory has previously shown that there is a progressive increase of carcinomas in the hamster cheek pouch upon continued DMBA treatment (9). The current finding that there is a significant association of infiltrating eosinophils with the later time points of DMBA treatment [weeks 12–16 (Table 1; Fig. 2)] prompted us to test the hypothesis that eosinophilia is associated with malignant oral epithelium. This was tested by examining tumor and non-tumor (normal/hyperplastic-dysplastic) areas in hamster cheek pouches treated with DMBA for 12–16 weeks. Eosinophilia is defined as ≥10 eosinophils/×200 field.

A total number of 15 different tumor-bearing hamster cheek pouches (weeks 12–16 of DMBA treatment) were examined. In these 15 tumor-bearing pouches, 36 separate tumors were examined. One non-tumor area from each pouch was also analyzed for tissue eosinophilia. Eosinophilia is defined as ≥10 eosinophils/×200 field. Of the 36 carcinomas examined, 28 (78%) exhibited adjacent eosinophilia, while only 1 of the 15 (7%) non-tumor areas had eosinophilia. A typical DMBA-induced hamster oral carcinoma exhibiting stromal eosinophilia is shown in Fig. 1A. Note the intimate association of the infiltrated eosinophils with the other inflammatory cells, in particular the macrophages, which are often laden with hemosiderin (Fig. 1B).

In order to test if the association of eosinophilia with malignant oral epithelium is statistically significant, the Fisher's exact probability test was used. Analysis revealed a significant association of tissue eosinophilia with malignant hamster oral epithelium when compared with non-tumor areas, beyond the 1% level.

**Expression of TGF-α mRNA by Infiltrating Eosinophils into DMBA-induced Hamster Oral Carcinomas.** We have recently demonstrated that eosinophils represent a major source of the cytokine TGF-α in hamster oral cancer (3). Approximately 90% of the infiltrating eosinophils were found to contain detectable cytoplasmic TGF-α mRNA by *in situ* hybridization. Our current finding that the eosinophil is a normal resident cell of the hamster cheek pouch lamina propria and that there is a significant association of tissue eosinophilia with malignant hamster oral epithelium prompted us to examine the following two issues: (a) do eosinophils express TGF-α constitutively (in normal and tumor-bearing hamster cheek pouches); (b) does the expression of TGF-α vary according to the association of eosinophils with either tumor or non-tumor hamster oral epithelium?

Seven normal and nine tumor-bearing hamster cheek pouches (DMBA-treated for 12–16 weeks) were subjected to TGF-α mRNA labeling by *in situ* hybridization, using sense and antisense 35S-labeled hamster TGF-α riboprobes (3, 8). Each hamster cheek pouch was examined at ×400, coupled with rhodamine fluorescence, for quantification of eosinophils and for eosinophils containing TGF-α mRNA. Ten such ×400 fields were examined for each pouch. For tumor-bearing pouches, examination of both non-tumor oral epithelium (normal/hyperplastic-dysplastic) and carcinoma areas were performed. It is important to note that even in hamster cheek pouches that have been treated with DMBA for 12–16 weeks, the entire pouch is not malignantly transformed (9). Week 12–16 DMBA-treated hamster cheek pouches contained ~20% normal and ~20% hyperplastic-dysplastic epithelium. Table 2 summarizes the data collected from the examination of these pouches. Fig. 3 is the graphic representation of the TGF-α mRNA-containing and -noncontaining eosinophils in normal cheek pouch, as well as in non-tumor and carcinoma-associated areas in week 12–

---

**Table 1** Quantification of eosinophils infiltrated into DMBA-treated and control hamster cheek pouches

<table>
<thead>
<tr>
<th>Wk of treatment</th>
<th>Control*</th>
<th>DMBA*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.5 ± 1.3</td>
<td>NA</td>
</tr>
<tr>
<td>6</td>
<td>0.95 ± 1.9</td>
<td>40.1 ± 30.6</td>
</tr>
<tr>
<td>8</td>
<td>17.6 ± 12.4</td>
<td>32.9 ± 11.9</td>
</tr>
<tr>
<td>10</td>
<td>78.8 ± 61.3</td>
<td>171.7 ± 173.3</td>
</tr>
<tr>
<td>12</td>
<td>31.1 ± 9.9</td>
<td>199.4 ± 98.2</td>
</tr>
<tr>
<td>14</td>
<td>24.5 ± 28.9</td>
<td>208.3 ± 86.3</td>
</tr>
<tr>
<td>16</td>
<td>14.4 ± 7.6</td>
<td>189.6 ± 157.0</td>
</tr>
</tbody>
</table>

* Four pouches from each time points were quantified for infiltrating eosinophils by counting 10 contiguous fields of each pouch at ×200. Data from the four animals in each group were pooled; mean ± SD is given. NA, not applicable.
16 DMBA-treated tumor-bearing hamster cheek pouches.

Similar to the previous finding, the number of eosinophils infiltrated into 12–16-week DMBA-treated hamster cheek pouches is significantly higher than the number present in normal cheek pouches (10.7 ± 9.2 versus 0.3 ± 0.5/×400 field; P < 0.01). No TGF-α mRNA containing eosinophils were detected in seven of the normal hamster cheek pouches examined (a total of 70 ×400 fields), despite the fact that eosinophils could be detected sparsely distributed in the lamina propria. In hamster cheek pouches that were treated with DMBA for 12–16 weeks, the number of eosinophils associated with tumor epithelium is significantly higher than those associated with non-tumor hamster oral epithelium (10.7 ± 9.2 versus 0.8 ± 0.6/×400 field; P < 0.001). The mean percentage of TGF-α mRNA-containing eosinophils is similar in tumor and non-tumor-bearing areas (80.7 and 77.1% respectively). However, the number of eosinophils labeled for TGF-α is significantly higher in carcinoma than in non-tumor areas (8.6 ± 9.2 versus 0.5 ± 0.5/×400 field; P < 0.01).

**DISCUSSION**

This paper describes the association of the eosinophil with the chemically induced tumor development process using the cheek pouch of the Syrian hamster as an experimental model. The results presented permit the following conclusions. The eosinophil is a normal resident cell in the lamina propria of the hamster oral mucosa. Upon continued DMBA treatment of the hamster cheek pouch, there is a sequential and progressive infiltration of eosinophils into the area. The number of infiltrated eosinophils by week 12–16 of DMBA treatment is significantly higher than that seen in the corresponding mineral oil controls. The infiltrated eosinophils in week 12–16 DMBA-treated pouches were found to be associated with malignant oral epithelium in a significant manner. By in situ hybridization, eosinophils in normal cheek pouches were not detected to express TGF-α mRNA. Whereas 77.1 and 80.7% of eosinophils associated with non-tumor and tumor hamster oral epithelium, respectively, contained detectable TGF-α mRNA. The number of TGF-α mRNA-containing eosinophils associated with malignant hamster epithelium is significantly higher than that associated with non-tumor oral epithelium.

Our report demonstrated, in an animal tumor model, the sequence and kinetics of eosinophil infiltration into the tumordeveloping site. Our results are in agreement and further elaborated the published work of Healy (11) on the cellular reaction during carcinogenesis. The end point of this tumor induction model is consistent tissue eosinophilia associated with malignant oral epithelium, similar to tissue eosinophilia seen in various human neoplasms (1). It is equally important to note that eosinophils are normal resident cells of the hamster oral mucosa, normally present in small numbers only. The resultant tissue eosinophilia observed subsequent to continued DMBA treatment is likely due to recruitment of this granulocyte from the peripheral blood. The biological signals responsible for such recruitment processes remain to be identified. Such eosinophilia could be (a) a non-specific "reactive" phenomena to the tissue injury, (b) a specific reaction to the carcinogen, (c) specific response to the carcinomas, or (d) a combination of these factors. It should be noted that eosinophilactic substances have been isolated from human large cell carcinoma of the lung (10).

Healy (11) and Flynn et al. (12) demonstrated the sequential infiltration of mast cells into the DMBA-hamster oral cancer model. Unlike the eosinophils, mast cells are present in large numbers in normal hamster cheek pouches (~40/mm²). Upon continued DMBA treatment, the number of mast cells increased but did not show a relationship to cancer development as is seen for eosinophils (12). Much research has demonstrated the
intimate relationship between eosinophils and mast cells (1). Mast cells have been hypothesized to release factors, such as eosinophil-chemotactic factor of anaphylaxis, which attract eosinophils into tissues (1).

There is large variance in eosinophil number and TGF-α-containing eosinophils among the pouches from the same treatment groups during our quantification process. The distribution of eosinophils in treated pouches is often patchy and scattered. Examining different portions of the same tumor can result in different patterns of eosinophil association. More importantly, eosinophils were found to contain different cellular contents of TGF-α mRNA in the same lesion. TGF-α mRNA-containing and -noncontaining eosinophils are often seen adjacent to each other. This suggests that the eosinophil is a heterogeneous population of cells that might be regulated in their activities by factors such as microenvironmental stimuli. Eosinophils in normal hamster cheek pouches were not detected to contain TGF-α mRNA. These data suggest that the expression of TGF-α by hamster eosinophils could be associated with the tumor development process. However, the stimuli for such an activation process might be either nonspecific (reactive) or specific to the carcinogenesis process.

The work presented in this paper depended on the detection of TGF-α mRNA by in situ hybridization to implicate the expression of TGF-α by the infiltrated eosinophils. We have previously demonstrated that ∼40% of the infiltrated eosinophils into hamster oral cancer sites contained detectable TGF-α protein evidenced by immunohistochemistry using a monoclonal antibody directed against the COOH terminus of the mature TGF-α peptide (3). Thus the production of TGF-α by the infiltrated hamster eosinophils has been established. Quantification of infiltrated eosinophils containing TGF-α protein, however, cannot be performed as precisely as that was done for detecting TGF-α mRNA by in situ hybridization. Scoring of infiltrated eosinophils for TGF-α mRNA by autoradiography followed by fluorescent microscopy for eosinophil identification can be done with a much higher level of confidence than scoring infiltrated eosinophils for TGF-α protein by the color change resultant from immunohistochemistry followed by fluorescent microscopy for eosinophil identification.

Tissue eosinophilia has been shown to be associated with a number of human carcinomas (13–19). More importantly, the presence of tissue eosinophilia has been suggested as a favorable prognostic indicator in cases of human neoplasms including head and neck cancers (13). These findings might support the hypothesis of a “protective” role of eosinophils and/or TGF-α in cancer. A recent report by Tepper et al. (20) demonstrated the in vivo antitumor effect of interleukin 4 is mediated by a host inflammatory infiltrate composed primarily of eosinophils and macrophages. However, it should be obvious that much in these hypotheses (tumor-associated eosinophils as protective, destructive, both, or neither) remains speculative. The availability of an animal tumor model demonstrating tissue eosinophilia parallel to tumor development will permit such hypotheses to be tested.

The data in this paper suggest that eosinophils are recruited into hamster oral cancer-developing sites and are preferentially associated with malignant epithelium. The majority of these infiltrating eosinophils are expressing the multifunctional cytokine TGF-α which can mediate a number of biological activities (mitogenesis, angiogenesis, bone resorption) and which, collaborating with other cells, can influence the process of tumor development (21). The demonstration that hamster eosinophils infiltrate into oral cancer sites in a fashion similar to that seen in human solid tumors supports the usage of this animal oral cancer model to study the role of this cell and its production of TGF-α in the malignant transformation process.

REFERENCES

Eosinophils, Tissue Eosinophilia, and Eosinophil-derived Transforming Growth Factor α in Hamster Oral Carcinogenesis

Mohammad Ghiabi, George T. Gallagher and David T. W. Wong


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/52/2/389

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.