

Fibroblast growth factor-2 expression during experimental oral carcinogenesis. Its possible role in the induction of pre-malignant fibrosis

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BACKGROUND: The fibroblastic growth factor (FGF)-2 has been shown to induce angiogenesis in several tumor types. To date, the activity of FGF during the development of oral pre-cancerous lesions has not been analyzed. We herein evaluated the role of FGF-2 in the pre-cancerous and cancerous lesions in the hamster cheek pouch oral cancer model.

METHODS: Expression of FGF-2 and its receptors FGFR-2 and FGFR-3 was assessed by immunohistochemistry at different stages of the carcinogenesis protocol. Activity of FGF-2 isoforms was analyzed by Western blots.

RESULTS: Increase and abnormal localization of FGF-2 expression was evident in cancerized epithelium before it was possible to detect morphologic alterations. The changes in FGF-2 are concomitant with the evolution of subepithelial fibrosis. Immunolabeling of carcinomas was faint or completely negative. Increases of FGF-2 activity are mainly due to the increase in the 18 kDa isoform. Receptors 2 and 3 of FGF are present in epithelium, fibroblasts, and vascular endothelia of control samples and in all stages of malignant transformation.

CONCLUSIONS: Our results would suggest a role for FGF-2 in the epithelium–connective interactions and a deregulation of its expression in the early stages of oral cancerization. In pre-cancerous tissue FGF-2 would play a central role in the development of fibrosis and a more collateral role in the induction of angiogenesis. The data would indicate its involvement in the process via the 18 kDa isoform.

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Introduction

The understanding of epithelium–connective interactions during the development of epithelial neoplasias is important in terms of the basic knowledge of the process and its implications in the control and prevention of malignant transformation. Among these interactions, tumor angiogenesis has been the most extensively studied. The induction of fibrosis has been less studied. Fibrosis differs from angiogenesis in that it is not constant for all tumors, despite the fact that it does characterize certain human neoplasias.

The relation between fibrosis and oral cancer is markedly evident in fibrosis of the submucosa, the well-known pre-cancerous lesion that is highly prevalent in India (1–4). The deep-rooted habit of the population of chewing betel that places the mucosa in direct and frequent contact with chemical carcinogens, is the proven cause of this phenomenon.

Similarly, in the most widely accepted animal model of oral cancer, the chemical cancerization of the hamster cheek pouch (5–8), the carcinogenic solution is spread over the mucosa. In this model, a marked desmoplasia is the first change observed underlying the cancerized epithelium, even before abnormal morphologic epithelial lesions occur.

Within the context of searching for factors that may be involved in the development of fibrosis, we studied the expression of fibroblastic growth factor (FGF-2) and its receptors. Despite the fact that the best-known function of this factor is the induction of angiogenesis (9–11), the presence of its receptors has been reported in fibroblasts, suggesting this factor may play a role in the synthesis of collagen. Fibroblast growth factors are involved in the transmission of signals between the epithelium and connective tissue, and influence growth and differentiation of a wide variety of tissues including epithelia (12).

The FGF-2, is one of the prototypes of the large family of growth factors that bind heparin. It is expressed in different tissues and has a wide scope of

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biologic activities (13). It binds to low-affinity heparan sulfate proteoglycans that are involved in the interaction with high-affinity receptors that in turn mediate the cellular response to FGF-2 (14, 15). The FGF receptor family consists of four members that have 55–72% amino acid homology (16).

The study of the expression of this factor in head and neck carcinomas (HNC) has yielded controversial results (17–19). The expression of FGF-2 and its messenger RNA has been reported to be erratic. The tumors that stain positively for FGF-2 exhibit heterogeneous expression that fails to correlate with clinicopathologic parameters (20). Other studies reveal increased expression of FGF-2 in well-differentiated HNC coupled to the presence of FGF-2 receptors. However, evidence is still insufficient to unequivocally attribute an actual role in tumor progression to FGF-2 (17). *In vitro* studies, however, have demonstrated the expression of FGF-2 in HNC where it is correlated with tumor growth (21).

Some aspects of the role of the growth factors in the process of carcinogenesis in the hamster cheek pouch model have been evaluated. In particular the role of the transforming growth factor (TGF)- α , a mitogenic cytokine whose main source are the eosinophils that infiltrate the areas that underlie transformed epithelia, has been investigated (22, 23). Epidermal growth factor (EGF) was also studied. While this factor was not detected in the early stages of cancerization, a positive reaction for its receptor (EGFR) was found (24). Very early changes in FGF-2 activity have been described during carcinogenesis in other animal models. Sumitomo et al. (25) reported an increased FGF-2 expression in hyperplastic and metaplastic epithelia associated with inflammatory areas in a model of rat submandibular cancerization. To date, there are no data on the association between the expression of FGF-2 and the different pre-neoplastic and neoplastic lesions in the hamster cheek pouch, a model which allows for the study of the development of non-inflammatory fibrosis during the process of carcinogenesis.

Materials and methods

Animals and tissues samples

Fifty-two Syrian hamsters, 6–7 weeks of age, 150–200 g body weight, were employed throughout. All procedures were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. The animals were submitted to a standard carcinogenesis protocol (5) that involves topical application of 0.5% dimethyl-1,2-benzanthracene (DMBA) in mineral oil, on the right cheek pouch, three times a week. Groups of six animals were killed at the end of weeks 6, 7, 8, 9, 10 and 16. The control groups comprised six hamsters treated with vehicle (mineral oil) alone and 10 untreated animals.

The pouches were everted and six samples were cut transversally from the extended pouch. Two samples were fixed in 10% neutral formalin, two samples were fixed in acetone and two were stored at -80°C , in

keeping with the requirements of the techniques described below.

The fixed material was embedded in paraffin. The formalin-fixed material was sectioned, stained with hematoxylin-eosin and employed for histologic analysis and selection of the areas to be evaluated.

Immunohistochemistry

The study of FGF-2 was performed on sections of tissues fixed in acetone, using a goat anti-FGF-2 polyclonal antibody (sc-79; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and a biotin-streptavidin-peroxidase kit (Biogenex, San Francisco, CA, USA).

The study of receptors 2 and 3 (FGFR-2 and FGFR-3) was performed on sections of formalin-fixed tissues which required antigen retrieval by incubation with 1 N HCl for 25 min at room temperature. FGFR-2 and FGFR-3 rabbit polyclonal antibodies (C-17 and C-15; Santa Cruz Biotechnology) were employed followed by incubation with Vectastain ABC system (Vector Lab., Peterborough, UK) FGF-2, FGFR-2, and FGFR-3 antibodies were diluted 1:100 in phosphate-buffered saline (PBS) from the original concentration of 200 $\mu\text{g}/\text{ml}$. Sections were counterstained with hematoxylin. Slides incubated omitting the primary or secondary antibody were employed to verify the specificity of the signal.

Quantitative evaluation of immunohistochemistry of FGF-2

Because control samples (both normal mucosa and mucosa treated with vehicle alone) showed a clear and homogeneous label in the basal layer and the process of carcinogenesis altered this pattern, we calculated the suprabasal labeling index (SLI) defined as: number of positive suprabasal cells divided by the total number of suprabasal cells in each selected area. Evaluation was performed with an image analyzer IBAS-Kontron (Jena, Germany).

In the epithelial cords of carcinomas, it was not possible to determine the value of SLI. We classified as positive when they exhibited at least one positive area and as negative when they failed to exhibit reaction.

Western blot analysis of FGF-2

Tissues samples were homogenized in buffer (5 $\mu\text{l}/\text{mg}$) containing 0.5 M Tris, 10% glycerol, 20% sodium dodecyl sulfate (SDS) and the protease inhibitors leupeptin (1 mg/ml), aprotinin (1 mg/ml), and phenylmethylsulfonyl fluoride (10 mM). Each cell extract corresponded to a single treated or normal pouch. Aliquots were taken for protein determination (DC Protein Assay, Bio-Rad, Richmond, CA, USA).

The protein extracts (70 μg of total proteins per lane) were prepared for analysis by dissolving them in denaturing buffer and resolved by SDS-polyacrylamide gel electrophoresis (PAGE) using a 15% gel. Non-specific sites were blocked with 5% dry milk. Blots were incubated with the anti-b-FGF antibody (sc-79, rabbit IgG; Santa Cruz Biotechnology) at 1:1000 dilutions for 1 h at room temperature. The positive bands were

identified by chemoluminescence (ECL Plus Western Blotting detection reagent, Amersham Life Science, Piscataway, NJ, USA). To control the load of each lane, each membrane was incubated with a polyclonal antiactin antibody (actin, C-11; Santa Cruz Biotechnology). The experiments were repeated for four to six pouches corresponding to each experimental time.

As specific control of the primary antibody we employed a fusion protein of FGF-2 of 41 kDa (FGF-2-10-140; Santa Cruz Biotechnology) and FGF-2 purified from a bovine pituitary gland (F-3133; Sigma, St Louis, MO, USA).

Results

Light microscopy observations

In keeping with previous descriptions (5–8), the lesions in cancerized pouches closely mimic the development of pre-malignant and malignant lesions of the oral cavity. After 6–10 weeks of DMBA applications, epithelium exhibited foci of hyperplasia and dysplasia that we grouped together as pre-neoplastic lesions (PREN) that alternate with areas of epithelium with no unusual microscopic features (NUMF; 26, 27). In these areas, the connective tissue exhibited marked fibrosis that increases the thickness of the cheek pouch. The severity of the lesions increases with the experimental time. However, at the end of the process, tumor areas coexist with pre-neoplastic lesions interspersed with NUMF areas. This phenomenon resembles the process of ‘field cancerization’ that takes place in the human oral cavity (27, 28).

Fibrosis tended to decrease as the epithelial lesions progressed. Below microinvasive lesions, a loose connective tissue with abundant vascular proliferation progressively took the place of fibrosis. This tissue gives rise to the stroma of carcinomas at a later time.

Expression of FGF-2

The untreated mucosa and mucosa treated with mineral oil alone exhibited immunolabeling for FGF-2 only in the cytoplasm of the basal layer cells of the epithelium (Fig. 1a). In pouches that had been cancerized for 6–9 weeks we observed a marked alteration in the expression of FGF-2, i.e. suprabasal cells, isolated or in groups, were labeled in NUMF and PREN areas (Fig. 1b,c). SLI increases with the severity of lesions. Figure 2 shows the values of the SLI for NUMF and PREN areas. The value of SLI peaked at 7 weeks for NUMF areas, when an intense subepithelial fibrosis had developed. However, at the more advanced stages of carcinogenesis SLI labeling decreased. At this time, NUMF and PREN areas are scarce because most of them have progressed toward microinvasive lesions and the overall fibrosis of the pouch wall had consistently decreased.

In carcinomas, which appeared at 12–16 weeks, FGF-2 was expressed only slightly or not at all. Nine of the 14 tumors evaluated (64.3%) were completely negative. The remaining five exhibited a heterogeneous reaction that tended to be localized in the basal layer (Fig. 1d).

The Western blots revealed immunoreactivity for FGF-2 in normal mucosa and in mucosa treated with vehicle alone. We observed the presence of two bands of 18 and 24 kDa and, in some cases, a very faint band corresponding to a low-molecular weight protein (16 kDa). The cell extracts of the pouches treated for 6, 9 and 10 weeks revealed a marked increase in the intensity of the 18 kDa band. The 24 and 16 kDa bands exhibited varying intensity. Carcinomas exhibited a remarkable reduction in the expression of the 18 kDa isoform of FGF-2. Some cases expressed faint 24 kDa bands and the low-molecular weight isoform (16 kDa) was not expressed in any of the cases (Fig. 3).

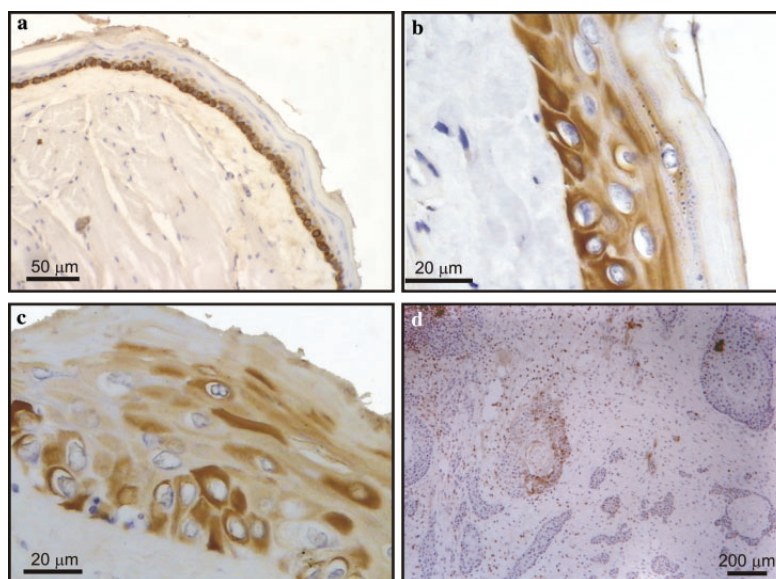


Figure 1 Immunohistochemical expression of fibroblastic growth factor (FGF-2). (a) Normal mucosa: positive reaction is restricted to the basal layer. (b) No unusual microscopic feature (NUMF) area, at 7 weeks of treatment, showing labeling in all the epithelial thickness. (c) A dysplastic pre-neoplastic lesions (PREN) area with inhomogeneous basal and suprabasal expression. (d) Squamous cell carcinoma exhibiting positive and negative areas (16 weeks of treatment).

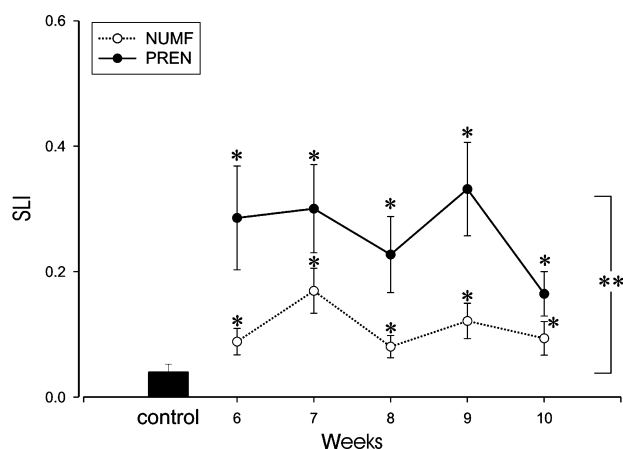


Figure 2 Quantitative evaluation of the immunohistochemical expression of fibroblastic growth factor (FGF)-2 using suprabasal labeling index (SLI) as the end point at intermediate cancerization times (weeks 6–10) for no unusual microscopic feature (NUMF) and pre-neoplastic lesions (PREN) areas. Two-way ANOVA, category factor $F_{1,148} = 26.8$, $**P < 0.0001$; time factor $F_{5,148} = 6.25$, $P < 0.0001$; interaction $F_{5,148} = 1.92$, $P = 0.09$. Newman–Keuls as *post hoc* test $*P < 0.05$ vs. control. The differences between 8, 9 and 10 weeks for NUMF areas and between 7, 8 and 9 weeks for PREN areas were not statistically significant. The data are expressed as mean \pm SD.

Immunohistochemical expression of the FGF receptors

Normal mucosa expressed both receptors, FGFR-2 and FGFR-3, in the full epithelial thickness. In most cases FGFR-3 exhibited a more intense reaction in the suprabasal layers. In the underlying connective tissue the expression of the receptors was observed in vascular endothelia and fibroblasts. The labeling was localized in the cytoplasm (dotted appearance) or on the membrane (Fig. 4a,b). NUMF and PREN epithelia and their underlying connective tissue failed to exhibit significant changes in the immunolabeling of the receptors. Epithelial labeling in carcinomas was significant and was predominant in basal layers (Fig. 4c). The tumor stroma showed a clear reduction in expression of FGFR-2, and even showed completely negative areas. Conversely, FGFR-3 labeling was still intense in fibroblasts and endothelia of tumor stroma.

Discussion

Taken as a whole, our data evidence a role for FGF-2 in the epithelium–connective interaction in the hamster cheek pouch mucosa and its deregulation during the process of carcinogenesis. In normal conditions, we showed that basal epithelial cells produce FGF-2. The presence of its receptors 2 and 3 in epithelial cells, fibroblasts, and endothelia evidence its participation in normal epithelial growth and in the maintenance of connective tissue structures and of the vascular network.

In the early stages of carcinogenesis both the basal cells and the keratinocytes of all the epithelial layers participate in an increased synthesis of the factor, mainly of its 18 kDa isoform. This increase occurs even before the first epithelial alterations become visible,

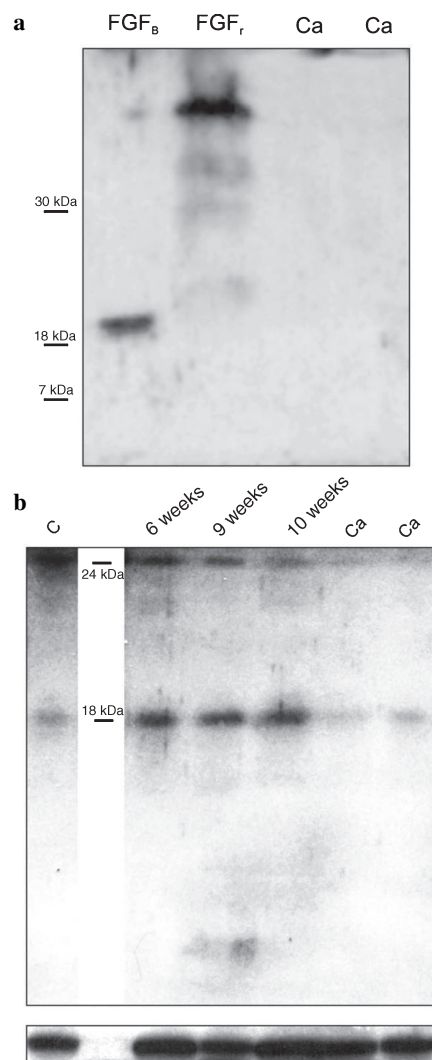


Figure 3 Detection of fibroblastic growth factor (FGF)-2 by Western blot analysis. (a) Positive controls – lane 1, FGF-2 of bovine pituitary gland; lane 2, FGF-2 fusion protein; lanes 3 and 4, absence of expression in two cases of carcinoma. (b) Lane 1, normal mucosa; lanes 2–4, pouches that had been cancerized with dimethyl-1,2-benzanthracene (DMBA) for 6, 9 and 10 weeks respectively; lanes 5 and 6, extracts of pouches cancerized for 16 weeks that had developed carcinomas. The bands corresponding to the 18 and 24 kDa isoforms of FGF-2 can be clearly observed in pre-cancerous tissues. Carcinomas exhibited a very faint expression. The detail below shows the internal control for each gel (actin).

when a marked fibrosis develops in the subepithelial connective tissue concomitant with the expression of receptors in fibroblasts and endothelia. However, a significant angiogenesis is not yet visible (see Light microscopy observations in the Results section). These results would evidence a role for FGF-2 in the early stages of carcinogenesis even when angiogenesis is not significant. The possibility that the 18 kDa isoform of FGF-2 should participate in the development of fibrosis is relevant in that varying degrees of desmoplasia are frequently associated with pre-malignant lesions of the human oral cavity.

Once the epithelial pre-malignant lesions appear, the number of suprabasal cells expressing FGF-2 continues

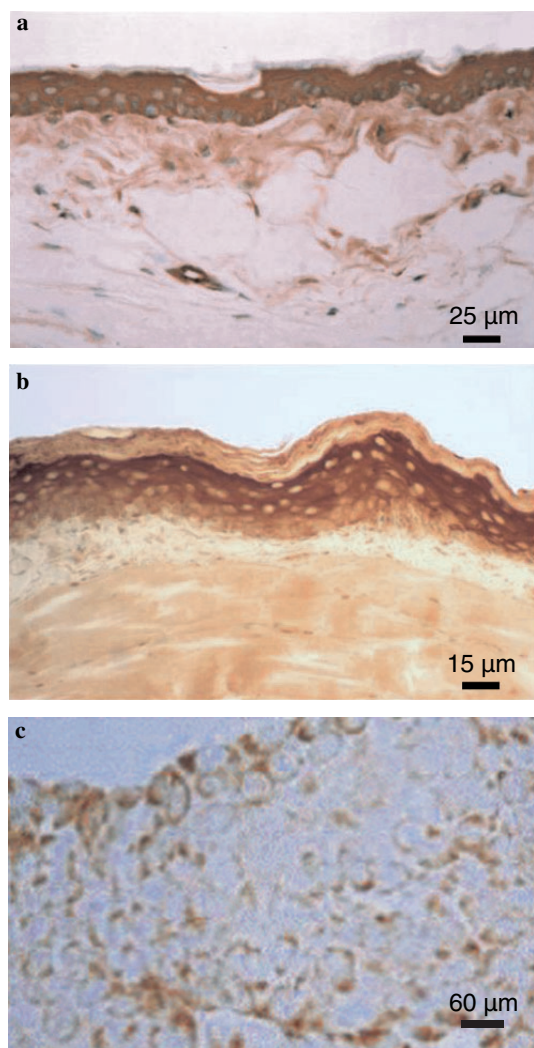


Figure 4 Immunohistochemical expression of fibroblastic growth factor receptor (FGFR)-2 and -3. (a) Normal mucosa that exhibited expression of FGFR-2 in the full thickness of the epithelium, endothelial cells, and fibroblasts. (b) Normal mucosa with stronger reaction for FGFR-3 in suprabasal epithelial layers. (c) Carcinoma exhibiting epithelial expression of FGFR-2 with an intense and patchy basal labeling.

to rise. Wakulich et al. (19) reported a similar pattern of behavior in human oral dysplasia. Our model allows for the sequential analysis of the different lesions as a function of time and revealed that the expression of FGF-2 in PREN areas decreases at longer cancerization times (nearly 4 months) when many of the lesions have begun their progression to carcinoma.

Most of the carcinomas fail to express FGF-2 and when they do, labeling is faint and heterogeneous. These variations in labeling are in keeping with the data reported by Janot et al. (17) for human carcinomas. We herein demonstrated that it is the 18 kDa isoform that greatly decreases its expression.

All of the carcinomas, even those that were FGF-2-negative, expressed both of the receptors evaluated. This finding is also in keeping with most of the data reported for human carcinomas. Some authors have assessed the

expression in tumors of the different variants of receptor 2. These studies reported that the variant of FGFR-2 that binds the keratinocyte growth factor (17–20) is predominant. The shift toward the expression of that isoform would explain the reduction in labeling for FGF-2 in oral carcinomas. However, this hypothesis would not be in keeping with the data of other authors who assessed the variant of receptor 2 that is specific for FGF-2. Wakulich et al. (19) reported the expression of FGFR-2 in all the epithelial layers of human dysplasia, and in carcinomas. These data agree with the results on expression of FGF-2. The tumor stroma in the hamster cheek pouch model does not exhibit areas of fibrosis, evidences a reduction in the expression FGFR-2 but continues to stain intensely for FGFR-3. Thus, the present study would show that the autocrine function of FGF-2, which is marked in pre-malignancy, would decrease once the process of malignant transformation has been completed. The persistent expression of the receptors, in particular FGFR-3, would evidence their participation in epithelial growth and angiogenesis via their interaction with another peptide of the FGF family.

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