Immunohistochemical Study on Epithelialization of the Fascial Flap in the Oral Cavity of Rats

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The immunohistochemical expressions of epidermal growth factor receptor (EGFR) and Proliferating Cell Nuclear Antigen (PCNA) were studied in the epithelialization process of bare flaps, the fascial flap and the deepithelialized mucoperiosteal flap, used for reconstruction of the oral mucosa in rat models. The intense expression of EGFR and PCNA was detected in the tip area of the migrating epithelium during the early period of the healing process in both flaps, and differing distributions of EGFR and PCNA positive cells were identified. In the fascial flap, a significantly high positive rate of EGFR was detected in comparison with the deepithelialized mucoperiosteal flap. The expression of EGFR in both flaps may be attributed to the influences of different connective tissue substrates which induce a control mechanism to maintain the equilibrium between differentiation and proliferation of epithelium.

Key words: **epidermal growth factor receptor (EGFR), mucosalization, fascial flap**

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Introduction

Injuries and ablative cancer surgery in the oropharyngeal passage result in mucosal defects, and skin flaps are widely used for the repair of these defects. Considerable attention has recently been given to fascial flaps without skin paddles, because these bare flaps mucosalize and resemble normal oral mucosa with minimal contraction in the oral cavity. The subcutaneous musculo-aponeurotic system (SMAS), commonly known as the superficial fascia, is thin, mobile, and well-vascularized. The SMAS has been reported to be successfully used as a reconstruction flap, fulfilling the functional requirements of the oral cavity (1-4).

Despite the number of reports based on clinical observation, the nature of the epithelialization of this fascial flap in the oral environment is poorly understood. In particular, there is almost no available information concerning the influence of fascial tissue substrates in terms of the effective regulatory mechanism for the epithelialization process of the fascial flap transferred into the oral cavity.

Wound healing is regulated by many substances, including cytokines and growth factors. Epidermal growth factor (EGF) and transforming growth factor-α (TGF-α) have been widely reported as being crucial to wound repair (5,6). As the mediators of the stimulation to keratinocyte growth, these growth factors have been most extensively studied. TGF-α and EGF exert similar biological effects by binding with the epidermal growth factor receptor (EGFR).

The present paper describes EGFR expression and proliferative activity in the epithelialization process of bare flaps, the SMAS and the deepithelialized mucoperiosteal flap in the oral cavity of rats.

Materials and Methods

Animals

The animals used in this study were 60 healthy male six week old Sprague-Dawley rats weighing 180-220 g. These rats were divided into two experimental groups of 30 rats each, A and B. In group A, a mucoperiosteal defect was made in the mandible and a pedicled cervical fascial flap was used to repair this defect. In group B, for the purpose of comparison, a deepithelialized mucoperiosteal flap was raised from the mandible and repositioned in situ.

Surgical procedure in group A

Animals were anesthetized by an intraperitoneal injection (40mg/kg body weight) of pentobarbital sodium, and the skin incision was made along the midline of the neck. In the oral cavity, an osseous exposure was made as a recipient defect on the superior and buccal aspect of the mandible in the edentulous region. An incision was

made along the alveolar ridge of the mandible from the first molar to the anterior tooth and was extended vertically across the lower lip to the inferior border of the mandible. The lower lip and buccal flap were raised up to the mental foramen, leaving the periosteum. The periosteum was excised anteroposteriorly from the vertical incision to the mental foramen and superioinferiorly from the longitudinal incision to the lower border of the mandible. This resulted in an osseous exposure about 5mm square in the edentulous region of the mandible.

The cervical fascial flap was designed based on vascular assessment by microangiography. The cervical fascial flap in the present experiment was fed by the fasciocutaneous branches of the superficial cervical artery branching from the subclavian artery.

In order to exhibit the cervical fascia of adequate size, the right side of the neck skin was separated from the underlying superficial fascia. The superficial fascia was separated from the underlying deep cervical fasciae, and was raised as a inferiorly pedicled cervical fascial flap parallel to the path of the feeder artery (Fig. 1a). The flap was long enough to cover the recipient defect without tension. The average size of the flap was about 1 cm in width by 3.5 cm in length.

This fascial flap was tunneled below the skin without tension to cover the bone exposure and sutured to the surrounding mucosa with 5-0 absorbable polyglactin suture (Ethicon, Inc., NJ, USA) (Fig. 1b). Finally, the skin incision was closed at the inferior border of the mandible to obtain a deep sulcus of buccal vestibule (Fig. 1c).

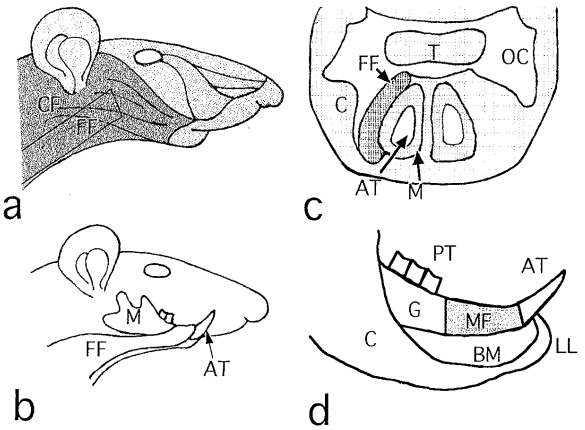

Fig. 1: (a). Design of the fascial flap (FF). (b). The fascial flap (FF) transferred into oral cavity. (c). Frontal view of the flap covering the mucoperiosteal defect. (d). Location of the deepithelialized mucoperiosteal flap (MF) in oral cavity. CF=cervical fasciae, M=mandible, AT=anterior tooth, T=tongue, C=cheek, OC=oral cavity, LL=lower lip, PT=posterior teeth, BM= buccal mucosa, G=gingiva

Operative technique in group B

To make the deepithelialized mucoperiosteal flap, in the oral cavity, the mucosa was deepithelialized approximately 5 mm in width by 5 mm in length from the alveolar ridge to the buccal sulcus in the mandibular edentulous region (Fig. 1d). The deepithelialized area was raised as a inferiorly pedicled mucoperiosteal flap. The pedicle portion of this flap was also dissected away from the bone down to the inferior border of the mandible, in order to produce a bone exposure similar to group A. This deepithelialized mucoperiosteal flap then was repositioned in situ and sutured to the surrounding mucosa.

Tissue preparation

All animals in groups A and B were perfused (n=5) with 4% paraformaldehyde for fixation sequentially under anesthesia at 3, 5, 7, 14, 21, and 28 days after the operation. The flap site of the mandible of each animal was sliced along the frontal plane, and was decalcified in 10% EDTA. Serial 4 μm sections were obtained from each specimen embedded in paraffin, and were stained with hematoxyline and eosin (HE).

Immunohistochemical staining

Immunohistochemical staining for EGFR and PCNA was performed by the avidine-biotin-peroxidase complex (ABC) method using Vectastain Elite ABC kit (Vector Lab., Burlingame, VT, USA).

The sections were incubated for 30 min in 0.3% H_2O_2 in methanol at room temperature to quench endogenous peroxidase activity. After rinsing with phosphate buffered saline (PBS, pH7.2), non-specific binding was blocked with 1.5% skimmed milk in PBS including normal blocking serum. Anti-EGFR and anti-PCNA antibodies were used as shown in Table 1. After being washed with PBS, sections were incubated for 30 min each with diluted biotinated secondary antibody and ABC reagent. They were then washed again in PBS, reacted with 3,3'-diamino-benzidine tetrahydrochloride (DAB), and counterstained with Mayer's hematoxylin.

Table 1: Primary antibodies used in this study

Antibody against	Type	Dilution	Source
EGFR	monoclonal	1 : 200	Sigma Chemical USA. Louis, MO,
PCNA	monoclonal	1 : 100	Novocastra Lab. Ltd., UK.

Sections from human squamous cell carcinoma of the oral mucosa were used as positive controls, since overexpression of EGFR has been reported (7).

The labeling index (LI) of EGFR and PCNA in the regenerated epithelium was calculated for every rat (n=5). In order to estimate EGFR LI, all the labeled and unlabeled keratinocytes in the regenerated epithelium were counted at a magnification of 400x. The counted area of the epithelium extended from the upper ridge of the alveolar bone to the migrating front for cases examined early in the postoperative period, and reached

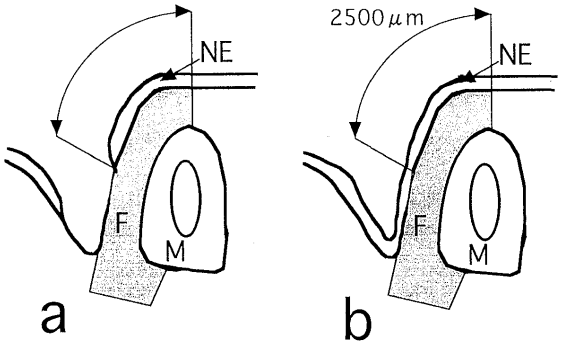

Fig. 2: (a). The counted area for 3,5,7 days after operation. (b). The calculated area for 21,28 days after operation. NE= newly formed epithelium, M= mandible, F= fascial flap or deepithelialized mucoperiosteal flap

Results

Flap survival and histological findings

The flaps in both groups survived without necrosis, and no exposure of the mandibular bone was seen in this study. In group A (fascial flap), the flap showed normal platysma muscle fibers with collagen fibers and little ground substance at 3 days after surgery. Acute inflammatory cell infiltration was found at the peripheries and the superficial portion of the flap. At 5 and 7 days, granulation tissue composed of collagen fibers and fibroblastic cells replaced almost the whole fascial flap (Fig. 3a). Acute inflammatory cells infiltrated the surface of the granulation tissue. At 3 days, the tip of the growing epithelium was seen just beginning to migrate

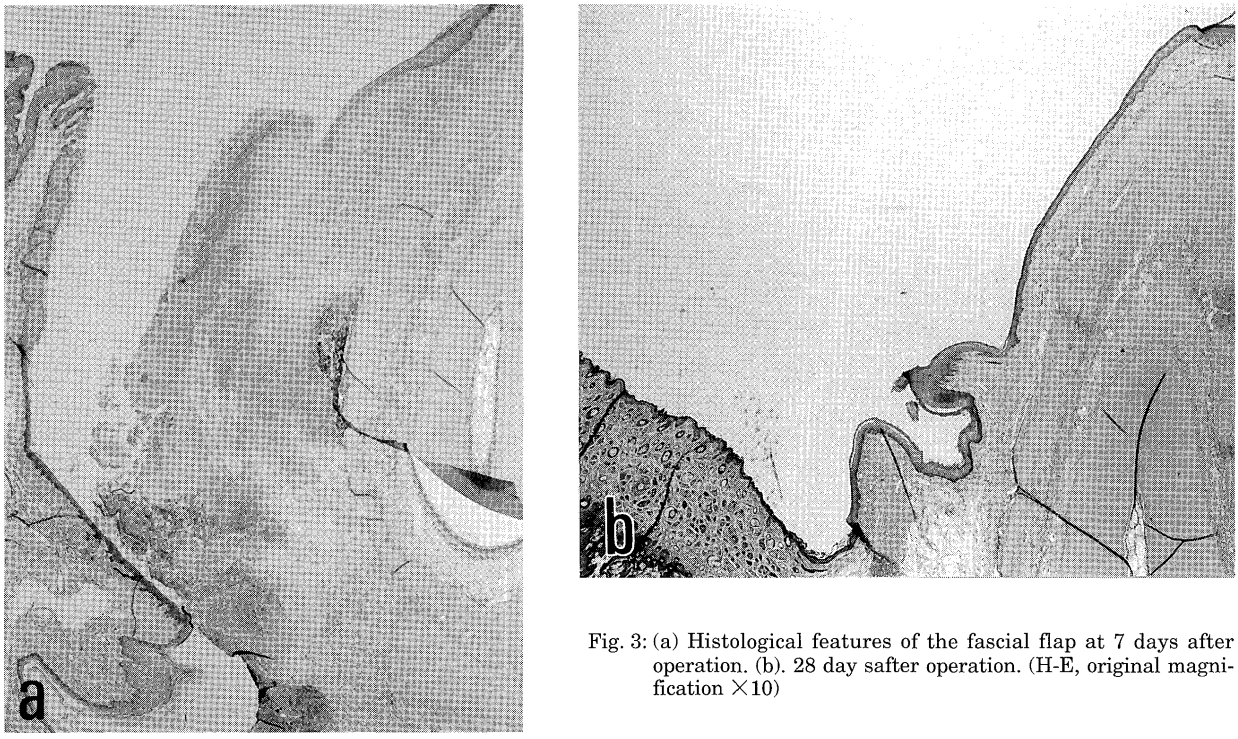

Fig. 3: (a) Histological features of the fascial flap at 7 days after operation. (b). 28 day safter operation. (H-E, original magnification ×10)

2500É m in length when the flaps were completely epithelialized at 21 and 28 days (Fig. 2). In order to estimate PCNA LI, labeled and unlabeled keratinocytes in the basal cell layer only were calculated in the defined area.

In the early postoperative stage, both EGFR and PCNA labeling indices (LIs) were also separately estimated in the tip area, extending 500 μm from the migrating front.

Statistical analysis

Results are expressed as means ± S.D. Statistical comparisons were made by analysis of variance and, when appropriate, using the unpaired Student t test with $P<0.05$. The nonparametric Mann-Whitney U tests for unpaired conparisons were also applied when appropriate.

into the flap area from the adjacent alveolar mucosa (Fig. 4a). At 5 and 7 days, the newly formed epithelial progress was seen to continue from the peripheral areas of the flap. The tip of the advancing epithelium was thin, and was migrating on the granulating fascial flap (Fig. 4b). At the same time, the epithelium adjacent to the epithelial tip stratified and showed increased thickness compared to the normal alveolar mucosa.

In group B (deepithelialized mucoperiosteal flap), the flap was also replaced by granulation tissue in these early postoperative stages. The histological features of the regenerated epithelium in group B were exactly similar to those of group A.

At about 14 days postoperatively, the granulating surface of the flap was completely covered by the epithelium in both groups A and B. At 21 days, the granulation tissue of both groups had maturated to fibrous tissue consisting of irregular collagen fibers, fibroblasts, and abundant blood vessels. In group B in particular, an

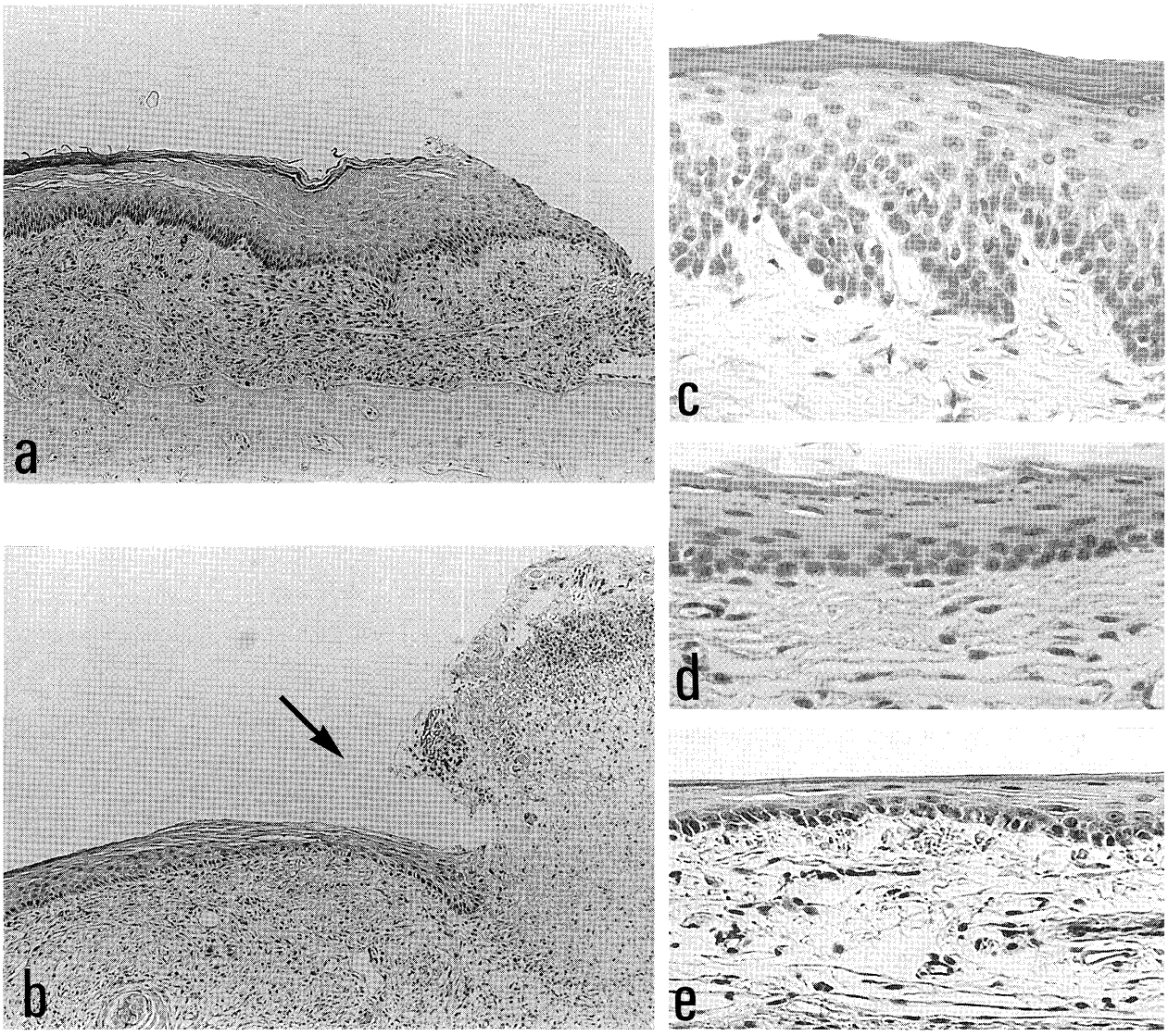

Fig. 4: Histological features of the epithelium on the fascial flap surface (H-E, original magnification ×25(a,b) ×100(c,d,e)). (a). the tip area of the migrating epithelium at 3rd postoperative day. (b). Epithelial migration on the granulating area of the fascial flap (arrow). (c). the irregular rete ridges of thick epithelium at 21 days after operation. (d). The new epithelium at 28 days. (e). the normal alveolar mucosa in the edenturous region.

increased fibrous component was observed, and the flap area and depth of the buccal sulcus tended to decrease. The newly formed epithelium was comparatively thicker than the normal epithelium in the edentulous region and had irregular rete ridges in both groups (Fig. 4c) .

At 28 days, the original surface area of the fascial flap was still almost completely preserved, the depth of the buccal sulcus was clearly maintained, and atrophy in the flap area was remarkable (Fig. 3b). In group B, the flap area showed severe contraction with the decreased depth of the buccal sulcus. The features of the epithelium of both groups closely resembled those of the normal oral mucosa (Fig. 4d,e). The stratified epithelium also showed decreased thickness and flat rete ridges similar to the original oral mucosa.

Immunohistological findings

In group A (fascial flap), the intense expression of EGFR was shown during the early period of the repair process ; i.e., at 3, 5 and 7 days after the operation. EGFR positive cells were mainly found in the tip area of the migrating epithelium and their distribution was predominantly observed in the suprabasal layers of the stratified epithelium (Fig. 5a,b).

At 21 and 28 days, very weak expression of EGFR was occasionally detected in the basal to prickle cell layer through the regenerated epithelium (Fig. 6a,b). These findings were also seen in the normal alveolar mucosa (Fig. 6c).

The distribution of EGFR positive cells in group B (deepithelialized mucoperiosteal flap) throughout the experiment was similar to that of group A.

PCNA positive keratinocytes in both flaps were revealed in the basal cell layer of the regenerated stratified epithelium throughout the experiment as well as normal mucosa. In particular, PCNA was strongly expressed in the tip area of the migrating epithelium in both flaps during the early stages of the epithelialization process (at 3, 5 and 7 days) (Fig. 5c,d). At 21 and 28 days, distribution of PCNA positive cells in both flaps

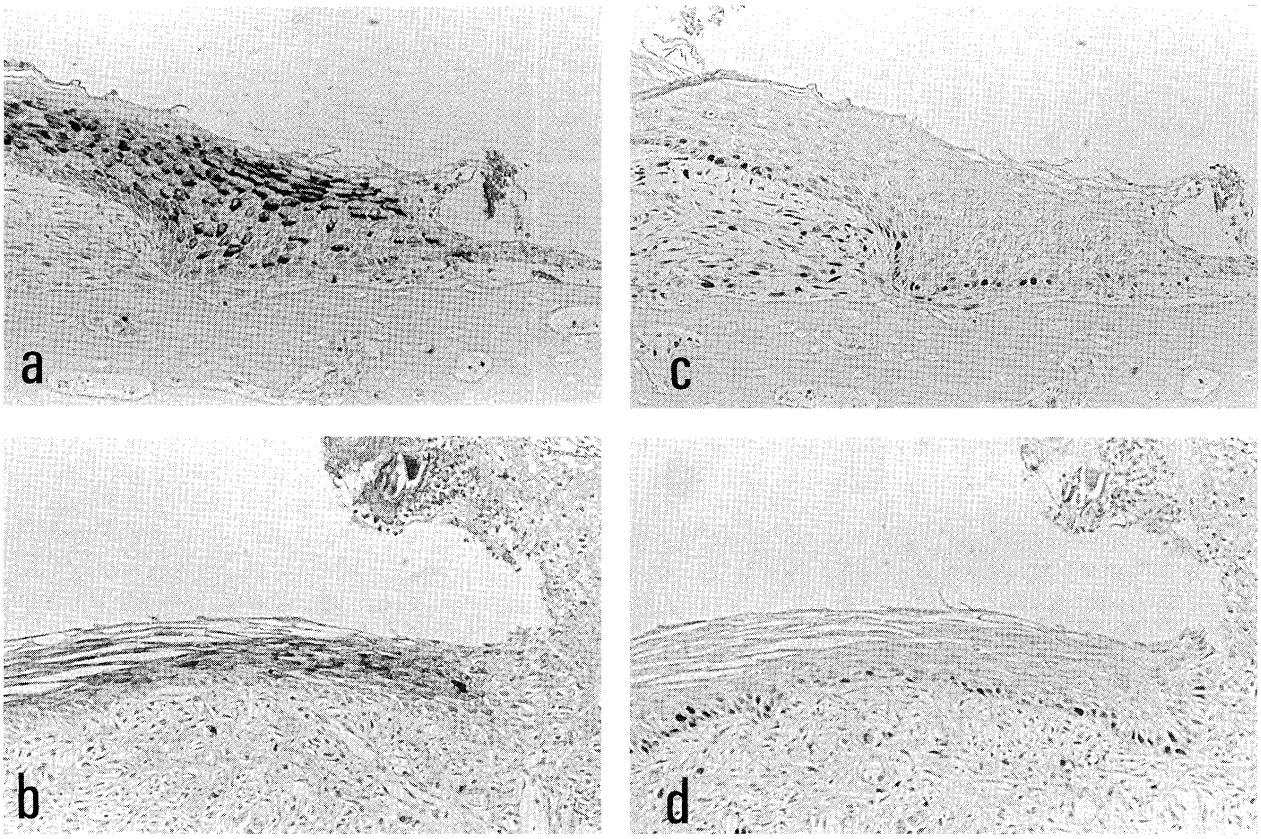

Fig. 5: Immunohistochemical staining. (a). At 5 and 7 days (b). postsurgery, EGFR positive cells locating in the suprabasal layer of the newly formed advancing epithelium. In the serial sections at 5 days (c) and 7 days (d), the basal layer cells are almost positive for PCNA. (original magnification ×100)

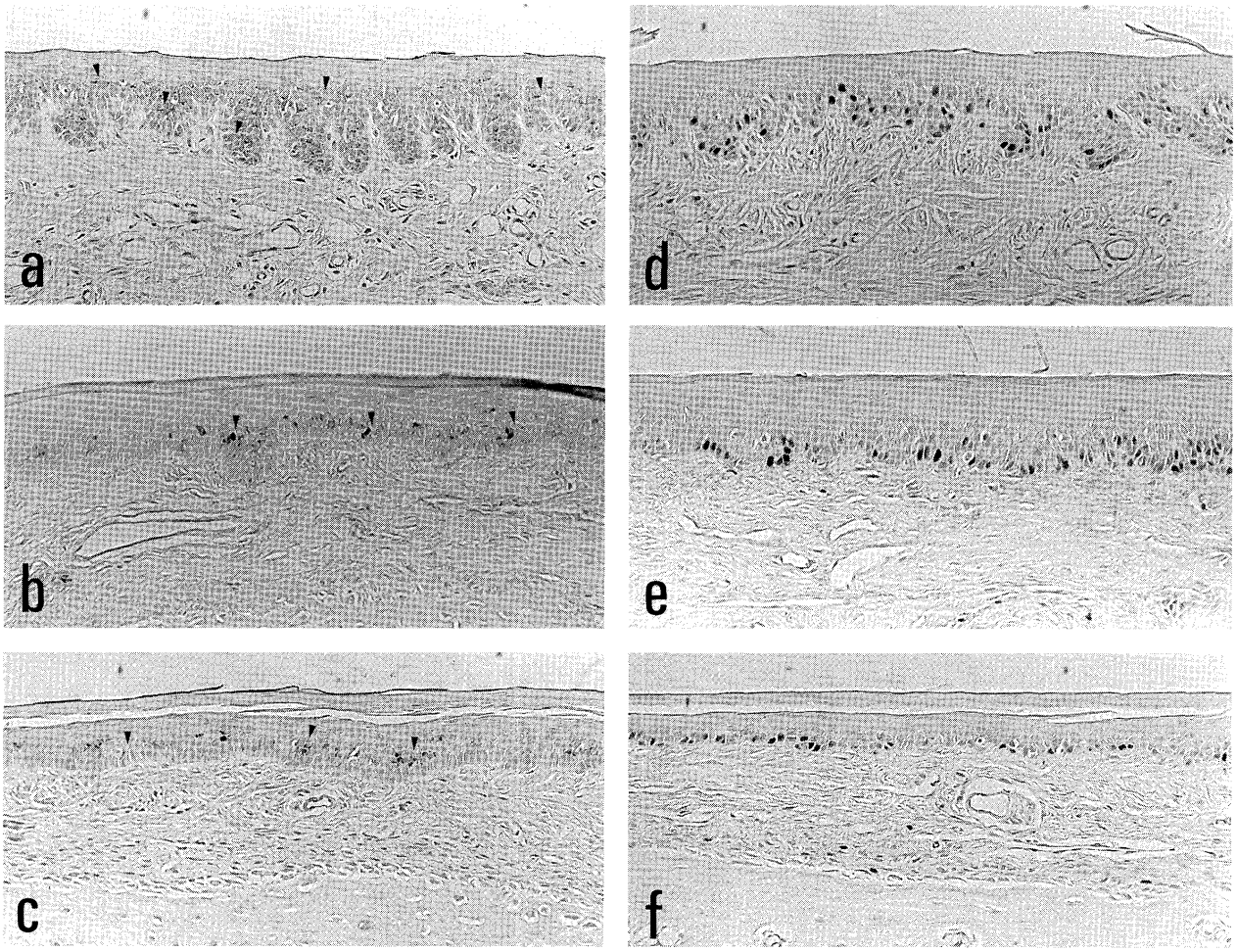

Fig. 6: Weak expression of EGFR (arrow heads) is occasionally detected in the basal and prickle cell layers at 21 days (a), 28 days (b) and in the normal oral mucosa (c). PCNA expression at 21 days (d), 28days (e) and in the normal mucosa (f). (original magnification ×50)

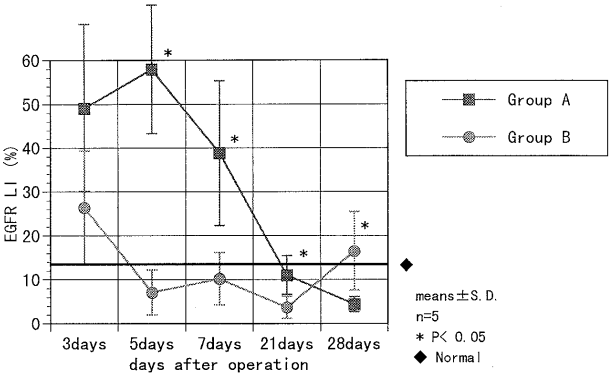

Fig. 7: (a). EGFR labeling index (LI) in the total area of the regenerated epithelium.

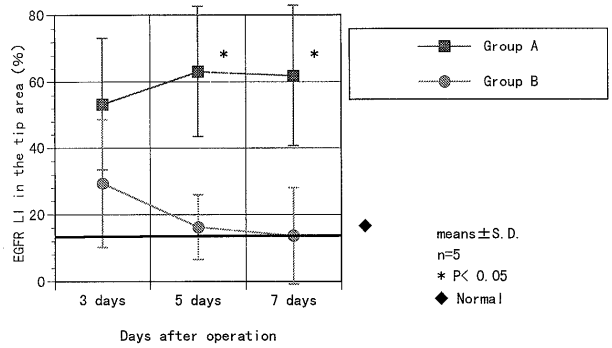

Fig. 7: (b). EGFR LI in the tip area of the regenerated epithelium.

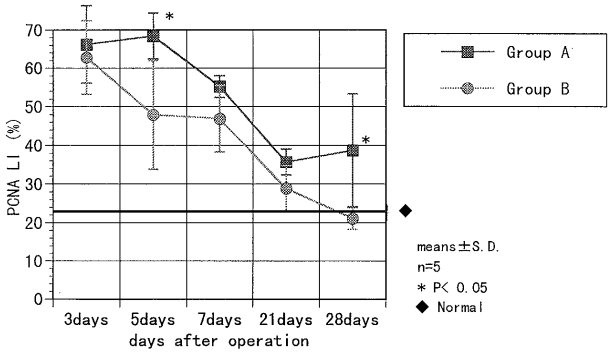

Fig. 8: (a). PCNA labeling index (LI) in the total area of the regenerated epithelium.

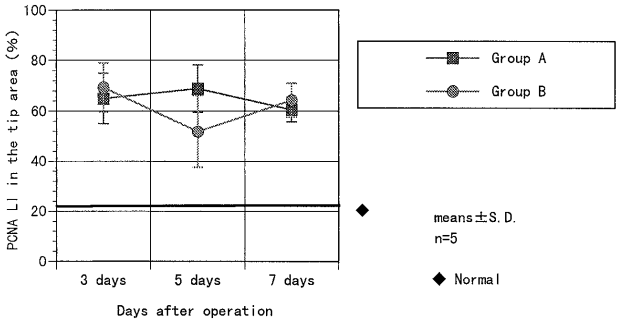

Fig. 8: (b). PCNA LI in the tip area of the regenerated epithelium.

was similar to that in the normal epithelium (Fig. 6d,e,f).

The EGFR and PCNA LIs of the fascial flap were compared with those of the deepithelialized mucoperiosteal flap. As shown in Fig. 7a, the EGFR LI of the total area of the regenerated epithelium in group A increased rapidly after the operation and reached its peak at 5 days postoperatively, thereafter decreasing rapidly until completion of epithelialization. In contrast, the EGFR LI of this area in group B gradually decreased following a peak at 3 days after operation. Statistically significant EGFR LI differences between group A and B were observed at 5, 7, 21, and 28 days. At the tip area of the regenerated epithelium in the early stages, a high level of EGFR LI was shown in group A (Fig.7b). The level of the EGFR LI of the tip area at 5 and 7days for group A was found to be statistically significant compared with the group B.

The PCNA LIs of the total area of the regenerated epithelium gradually declined through the experimental course (Fig. 8a). The high average PCNA LI of group A at 5 and 28 days was statistically significant compared with that of group B. The PCNA LIs of the tip area in both groups (Fig. 8b) were higher than that of the total area. No significant differences in PCNA LI were observed between group A and B in this tip area.

Discussion

The serial events of wound healing, including inflammation, fibrosis, epithelialization, angiogenesis and tissue remodeling, were shown in our experiments. Wound healing has been also reported as a dynamic physiological process that is affected by many contributory factors (8). The granulation tissue appearing in the healing process consists of immature extracellular matrix and fibroblast secreting a large amount of cytokines and growth factors. Growth factors are produced locally at the wound site and control the supply of new cells and the formation of new extracellular matrix, thereby accelerating or slowing wound repair (9-11). EGF and TGF-α have been the most extensively studied as the mediators of stimulation to epithelial growth, and have been found widespread biological effects on various cells by binding with EGFR (5,12-14).

It was speculated by the authors that the distinctive expression of EGFR, one of the regulatory elements in the epithelialization process, could be especially identi fied in the fascial flap than in the normal mucosa, since the origins of the tissue component of the fascial flap are different from those of the oral mucosa. In our experiment, therefore, the bare deepithelialized mucoperiosteal flaps was also investigated as a model for comparison with the fascial flap.

Previous reports have shown that EGFR positive cells were distributed only in the basal cell layer of the normal human skin and oral mucosa (15,16). In contrary, EGFR have been shown throughout all layers in stratified epidermis in burn wounds during early

postburn period (17). Furthermore, in acquired skin lesions such as seborrheic keratosis, EGFR is exhibited in all the epidermal layers in rapidly proliferating lesions, while it is limited to basal keratinocytes in slow-proliferating lesions (18). In the present study, the high level of PCNA LI observed in the tip area of the regenerated epithelium in both experimental groups during the early healing stage indicates increased proliferating activity, which was observed to result in epithelial cell stratification. It is interesting that EGFR positive cells mainly appeared in the prickle and granular cell layers in this area and were negative to PCNA expression in both flaps. The localization of EGFR in the suprabasal cell layers observed in the present study seems to indicate a characteristic feature of the rapid epithelial regeneration. In addition, based on a report by Barrandon *et al.* (19) suggesting that rapid resurfacing of wounds could be due to an increased rate of cell migration after EGF exposure, it would also be possible that the localization of EGFR shown in the present study is associated with epithelial cell migration or maturation along with epithelial cell stratification during rapid regeneration.

While there was a significant difference in the EGFR LIs between the fascial flap and the deepithelialized mucoperiosteal flap, the PCNA LIs were not significant. This contrast may result from the difference in the subepithelial connective tissue component between the fascial flap and the deepithelialized mucoperiosteal flap. Subepithelial connective tissue has been suggested to play an important role in supporting epithelial proliferation and differentiation known as epithelial-mesenchymal interaction (20,21). In particular, growth factors released from the underlying connective tissue are reported to induce various inhibitory or stimulatory responses depending on the cellular environment during the process of tissue repair (11,22).

The contractile feature was shown to be different between the fascial flap and the deepithelialized mucoperiosteal flap. With regard to the contraction of flaps, a previous study undertaken by our department showed that the muscle flap epithelialized with severe contraction compared with the fascial flap (23). The distinction concerning contraction between the flaps in the present study may also be due to the different substrates of the flaps, including the extracellular matrices. However, the interaction between extracellular matrices and epithelialization or tissue contraction has not yet been clarified, and further investigation will be needed to confirm the influence of the connective tissue substrates including growth factors in the healing process of the flaps.

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