Original Article

Preservation of rat palatal scar tissue myofibroblasts in organ culture

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In order to modulate palatal scar tissue, especially its myofibroblastic component, there is a pressing need for an in vitro model of this tissue. In the present, study we established an organ culture model of the rat palatal scar tissue. After excision of palatal mucoperiosteum, explants from the developing immature scar tissue and from the normal palatal mucosa were used to observe myofibroblasts in vivo and their maintenance in organ culture. Explants were cultured at the gas-liquid interface in serum-free Waymouth’s MB 752/1 medium and in a humid atmosphere containing 55% O2/5% CO2 in air at 37 °C for 3 days. Viability of the cultured explants was evaluated with morphological and histological criteria and BrdU incorporation. After organ culture, the scar tissue showed good preservation of the in vivo histology. The myofibroblasts and smooth muscle cells of the cultured scar tissues showed continuous expression of alpha-smooth muscle actin (α-SMA), mimicking the in vivo situation. In the normal tissues, only smooth muscle cells of the blood vessels expressed α-SMA. These results demonstrate that the established model provides a useful in vitro experimental tool for investigating the palatal scar tissue in general and its myofibroblasts in particular.

Key words: Palate; Scar; Myofibroblast; Organ culture; Rat

Introduction

The scar tissue that develops after surgical closure of cleft palate is a major cause of both growth distortion in the maxillofacial skeleton and relapse after orthodontic maxillary expansion. This sequence of events is well described in animal studies12 and clinical reports3-4. It is an important issue to gain an understanding of the molecular and cellular mechanisms by which the scar tissue functions in order to develop efficient therapeutic interventions.

Palatal scar tissue is the end result of the healing process, which generally can be divided into processes of epithelization, contraction, collagen synthesis, and scar remodeling5. The ability of palatal scar tissue to cause growth disturbance and dentoalveolar sequelae is attributed to the excessive formation of this tissue5, and, in particular, to the presence of numerous myofibroblasts during the healing process of the palatal wounds6. The myofibroblast cell is considered to be a key cell that plays a fundamental role in the wound healing and scar formation process. It is believed that the myofibroblast with its content of alpha smooth muscle actin (α-SMA) provides the contractile element responsible for wound contraction7,8. Additionally, this
cell is active during extra-cellular matrix deposition and collagen synthesis in response to growth factors secreted in the wound environment such as transforming growth factor-β (TGF-β)².

Various in vitro systems ranging from monolayer culture to three dimensional collagen gels have been used for investigate this cell type and its regulation by different factors. Studies using these culture systems in our laboratory have also shown that fibroblasts isolated from rat palatal scar tissue and TGF-β1-pretreated normal palatal fibroblasts both possess myofibroblastic characteristics from morphological and biochemical standpoints. Now to move the study of palatal myofibroblasts from the cellular level to the tissue level for the purpose of modifying them in light of the recent use of recombinant cytokine therapy to modulate scarring of wounds, there is a need to establish an in vitro organ culture model of the palatal scar tissue. Hill and Miles have successfully established a rat normal palatal mucosa organ culture model, but there has been no reports regarding the palatal scar tissue in organ culture. Therefore in this study, we took advantage of the organ culture method that provided conditions close to the in vivo situation but separate from systemic factors to established a short-term organ culture model of the rat palatal premature scar tissue with special emphasis on its myofibroblastic component.

Materials and Methods

Scar tissue and explants preparation

A full thickness excision of palatal mucoperiosteum (2x4 mm) was performed in 8-week-old Sprague-Dawley rats under ether anesthesia. After 4 weeks, explants of the same size were dissected from the developed immature scar tissue and from the normal palatal mucosa of age-matched animals. The explants were washed first in sterile Hanks’ balanced salt solution (GIBCO, Grand Island, NY) and then they were washed in the sterile culture medium before they were cultured. Another set of explants from both tissues was processed directly for histological procedures and considered as non-cultured control.

Organ culture technique and evaluating criteria

Explants from both tissues, normal mucosa and scar tissue, were placed epithelium surface side up on 0.4-μm-pore inserts of 30 mm diameter (Millipore Corp., Bedford, MA). One or two explants were cultured in a single insert. The inserts were placed in 6 well-culture plates (diameter 35 mm) containing 1.2 ml of the culture medium in each well. This method kept the cultured explants at the gas-medium interface without submerging them (Fig. 1). The culture medium was composed of serum-free Waymouth’s MB 752/1 medium (GIBCO BRL., Grand Island, NY) supplemented with 1% penicillin-streptomycin solution (GIBCO BRL.), 2 mg/ml bovine serum albumin (SIGMA CHEMICAL CO. St. Louis. MO), 0.825 μg/ml FeSO₄.7H₂O, 300 μg/ml ascorbic acid, and 1 μg/ml hydrocortisone sodium succinate (all from WAKO Pure Chemical Industries, Ltd. Japan). The medium was replaced every other day, and the organ cultures were incubated in a humid atmosphere containing 55% O₂/5% CO₂ in air at 37 °C for three days. Tissue explants were fixed in 10% neutralized buffered formalin, processed by the standard procedure for histology, and embedded in paraffin. Frontal sections 5 μm in thickness were stained with haematoxylin and eosin or were used for immunohistochemistry. The overall preservation and the viability of the organ-cultured explants were evaluated by using several criteria: (1) preservation of tissue architecture and morphology; (2) preservation of epithelium-connective tissue junction area; (3) epithelium keratinization, stratification, and migration of epithelial cells at the explant margins without complete encapsulation of the explants by the migrating epithelial cells; and (4) Morphology and densities of the lamina propria cells and fibers and preservation of blood vessels.

Immunohistochemical procedure

To examine the expression of α-SMA, sections were deparaffinized and rehydrated; endogenous peroxidase activity was quenched in 3% H₂O₂/methanol.
Fig 2. In vivo histological appearance and presence of myofibroblasts observation in palatal scar and normal palatal mucosa explants stained with haematoxylin and eosin (A) (B) (C) or with immune-stain for α-SMA (D) (E) (F). (A): palatal scar. (B): normal palatal mucosa. (C) shows the elongated shape of the scar myofibroblasts (MF; arrows). (D): α-SMA expression in the scar myofibroblasts (MF; arrows) and walls of blood vessels (BV). (E) α-SMA expression only in the blood vessels walls of the normal palatal mucosa. (F): Negative control of the scar tissue explant showed no staining. Original magnification × 50 (A, B), × 200 (C, D, E, F).
solution for 5 minutes. After washing with PBS, sections were treated with 1.5% normal horse serum in PBS for 20 minutes and then with anti-α-SMA monoclonal antibody (1A4, ARP, Inc., Belmont, MA) diluted at 1:100 in PBS for 1 hour at room temperature. After being washed, the tissue sections were incubated in biotinylated horse anti-mouse IgG (Vector, Burlingame, CA) followed by incubation with avidin-biotin-peroxidase complex (Vector, Burlingame, CA). Irrelevant mouse IgG (Sigma Chemical Co.) was used as the negative control. The sections were developed with 0.1% diaminobenzidine in PBS with 0.02% H₂O₂ and counterstained with methylgreen.

BrdU labeling of organ-cultured tissues

BrdU labeling was carried out using a ‘BrdU labeling and detection kit II’ from Boehringer Mannheim. Both tissue types, normal and scar explants, were labeled with BrdU (10 μM/l) during the last 20 hours of culture. The labeled samples were reincubated with the washing buffer of the same kit for 30 minutes at the same culture conditions mentioned above, fixed in 70% ethanol, paraﬃn embedded, and sectioned at 3 μm. BrdU. Incorporation into proliferating cells was detected by immunohistochemistry with a monoclonal antibody against BrdU, which was included in the kit.

Results

Non-cultured tissues

Four weeks after excision when the palatal wounds were completely healed, explants of both premature scar tissue and non-wounded normal tissue were observed by light microscopy for morphological and immunohistochemical differences with staining by haematoxylin and eosin or anti-α-SMA monoclonal antibody. The scar tissue (Fig. 2A) comprised of its two major elements, epithelium and connective tissue, was thicker than the normal tissue. The epithelium appeared with deep rete pegs penetrating into the stromal papillae in all areas of the scar tissue explants; but, in the normal explants (Fig. 2B), deep rete pegs were seen only in the palatal rugae where there was a notable increase in thickness of the epithelium. Therefore, to facilitate comparison, we chose sections from the inter-rugal regions of the normal explants. The connective tissue of the scar explants was different from that of the normal tissue. It appeared as a very dense structure, rich in thick bands of collagen ﬁbers, and showed an increase in the cell density. These cells appeared large by haematoxylin and eosin staining. They had an elongated shape and were distributed abundantly among the collagen ﬁbers as shown in Fig. 2C. By α-SMA-immune staining, many of these cells exhibited a clear expression of this protein as shown in Fig. 2D. Alpha-SMA was also present in smooth muscle cells in the walls of the blood vessels, which served as a positive control. Except for these vascular smooth muscle cells, alpha-SMA-positive cells were myoﬁbroblasts, which were oriented parallel to the palatal transverse plane. On the other hand, in the normal tissues, there were no α-SMA-positive myoﬁbroblasts, and the α-SMA expression was restricted to the vascular smooth muscle cells (Fig. 2E). Sections treated with mouse IgG as a negative control showed no staining (Fig. 2F).

Organ-cultured tissues

Four weeks after excision, explants from both the scar and the normal tissues were maintained in organ culture for 3 days. Both kinds of tissue explants were flattened and had become adhered to the filters without any adherence promoting materials. There was no change in the size or the shape of the explants in culture. The histological examination of both palatal scar tissue and normal tissue explants after 3 days in organ culture revealed maintenance of the overall structures of these tissues (Fig. 3A, B). The epithelium was still well organized with intact vital cell layers and regular stratification from basal cells through a prickle cell layer and a granular layer to the keratin layer. Mitotic ﬁgures were seen; however, a number of dyskeratotic cells containing densely staining nuclear remnants were seen within the epithelium. (Fig. 3C, D). At the connective tissue level also, the histological picture was similar to that of the in vivo control. The connective tissue of the scar explants maintained its dense structure over the culture period, and the ﬁbrous network along with those myoﬁbroblasts within it was highly preserved. By light microscopic observation, showed that the myoﬁbroblasts in the connective tissue of the scar explants were seen to have preserved their elongated shape (Fig. 3E). Continuous expression of α-SMA by these myoﬁbroblasts and by the smooth muscle cells of the blood vessels was also preserved. A good staining density was seen, and the myoﬁbroblasts appeared in the same orientation as those of non-cultured scar tissues (Fig. 3F). In the normal organ cultured-tissue explants, only smooth muscle cells of the blood vessels expressed α-SMA (Fig. 3G). Sections stained with mouse IgG as a negative control
Fig 3. Histological appearance and presence of myofibroblasts observation in the organ-cultured palatal scar and normal palatal mucosa explants stained with haematoxylin and eosin (A, B, C, D, E) or immune-stain for α-SMA (F, G, H).

Overall views show good preservation of the overall structure of both scar explant (A) and the normal tissue explant (B) after three days in organ culture. (C): organ cultured palatal scar. (D): organ cultured normal palatal mucosa. Note the mitotic figures (arrowheads) in the epithelium of both tissues. (E): shows the maintaining of the scar myofibroblasts (MF: arrows) in organ culture. (F): α-SMA expression in the scar myofibroblasts (MF: arrows) and the walls of blood vessels (BV) in organ culture. (G) α-SMA expression only in the blood vessels (BV) walls of the normal palatal mucosa in organ culture. (H): Negative control of the normal palatal mucosa in organ culture showed no staining. Original magnification × 12 (A, B), × 50 (C, D), × 200 (E, F, G, H).
showed no staining (Fig. 3H).

To assure the cell viability of the organ cultured tissues, we labeled them with BrdU. A number of epithelial cells in the basal cell layer incorporated BrdU in both normal and scar tissues (Fig. 4A, B). Furthermore, epithelial cells of the basal cell layer along the migrating epithelium actively proliferated in both tissues (Fig. 4C, D). The connective tissue cells also continued to divide during organ culture, as was shown by the BrdU incorporation (Fig. 4A, B). These results showed that epithelial and connective tissue cells continued to proliferate in this organ culture model.

Discussion

In the present study, premature scar tissue was induced in the rat palate following an experimental full thickness excision of mucoperiosteum. The morphological characteristics as well as the existence of myofibroblasts in this scar tissue were observed and compared with the normal palatal tissue. Further, we established a short-term organ culture model for this tissue using a serum-free medium and a stationary organotypic culture system by which we observed the maintenance of those myofibroblasts within their original tissue structure. We hope to use this model in the future to study the response of the palatal scar tissue to the proposed therapeutic factors in order to minimize the contraction of this tissue and to modify its myofibroblastic cells.

Myofibroblast is an altered phenotype of fibroblast with many biochemical, ultrastructural, and histological features similar to those of smooth muscle cells, These features include an extensive cytoplasmic fibilar system, complicated folds and indentations in the nuclei, and cell-to-cell and cell-to-stroma attachments. The most prominent biochemical feature of myofibroblast is its expression of the intracellular \( \alpha \)-SMA, which is therefore considered to be the best marker for distinguishing between the myofibroblast and normal fibroblast phenotypes. Myofibroblasts were first described within granulation tissue and were also found in different normal and pathological situations. These cells are known as the force generator in scars and fibrotic diseases accompanied with contraction. Their emergence in the palatal scar may explain the potential contraction force of the palatal scar since this tissue is one of the typical pathological situations with excessive contraction. This contraction is usually reflected by disturbance in maxillary growth and functional impairment manifested as partial or total cross bite as well as difficulties in orthodontic treatment. Most reports on palatal scar tissue have used the dog as an experimental model, whereas in this study we have used the rat as an animal model to address some histological and molecular aspects of this issue.

The haematoxylin and eosin staining of the in vivo explants in this study have shown the over all architecture of the premature scar tissue especially the connective tissue where myofibroblasts accumulated and appeared large and spindle shaped, the typical histological features of myofibroblasts mentioned by Schurch et al. The accumulation of myofibroblasts was confirmed by their expression of \( \alpha \)-SMA, which clearly showed their distribution. The role of the myofibroblasts in generating the contractile force of the palatal scar is suggested since these cells usually appear to be oriented parallel to the axis of the tension generated in the tissue. In our model we also observed narrowing of the intramolar width of the rat palate after scar formation (data not shown). This is in agreement with the notion that one of the most common sequelae of the palatal scar is the presence of partial or total cross bite. The maximum \( \alpha \)-SMA expression in our rat palatal scar model was observed to be maximum around 3 weeks after excision (data not shown). Nevertheless we chose the time point of 4 weeks for explanting this tissue, because, at this point, the tissue was more solid. At the same time, it was still in the premature stage and in the remodeling phase. These data confirm and extend our previous studies in which we described myofibroblastic characteristics of the palatal scar fibroblasts. Myofibroblasts differentiation and \( \alpha \)-SMA expression are believed to be regulated by many factors such as heparin, granulocyte macrophage-colony stimulating factor and TGF-\( \beta \)1. Palatal fibroblasts also have shown myofibroblastic phenotype modulation and upregulation of \( \alpha \)-SMA expression upon TGF-\( \beta \)1 treatment similar to that of myofibroblasts derived from the palatal scar tissue. On the other hand, down regulation of the \( \alpha \)-SMA expression in TGF-\( \beta \)1 pre-treated normal fibroblasts was induced by interferon-\( \gamma \), which altered the spindle shape of those cells into polygonal shape of the non-treated palatal fibroblasts. The palatal myofibroblasts disappear after finishing their mission to heal the palatal wounds by apoptosis and apoptosis, could be induced in these myofibroblasts in vitro by basic fibroblast growth factor treatment.

The in vivo myofibroblasts' appearance and disap-
Fig 4. Immune-stain for BrdU in the organ cultured palatal scar and normal palatal mucosa explants. BrdU incorporation is shown in the epithelium and the connective tissue of both scar explants (A) and normal tissue explants (B), (arrowheads). The proliferation of the migrating epithelium in the scar margins (C) and normal tissue (D) explants is clearly shown by BrdU positive keratinocytes (arrows). Original magnification × 100 (A, B) × 50 (C, D)
pearance seem to be regulated by complex co-operation of several factors. It is especially important to study these factors to gain a thorough understanding of the mechanism of these processes, but in vivo the complex nature of the wound microenvironment coupled with the interference of systemic factors make it difficult to obtain precise data. Conversely in vitro the myofibroblastic phenotype and α-SMA expression may be affected by the culture conditions such as the cell density and the deformability of the culture substrate. Therefore, we sought to establish an organ culture model for the palatal scar tissue at the time when this tissue is rich in myofibroblasts and during the remodeling phase. The organ culture technique has been a useful method for studying the behavior and permeability of the normal neonatal rat palatal mucosa in culture. Experiments looking at skin wound repair in vitro and at the responses of wounded tissue to pharmacological agents and growth factors usually employ this system as well. Most of the organ culture studies reported regarding the oral mucosa have used a Trowell-type method. In this study, however, we applied a relatively simple stationary-type technique of organ culture that enabled us to keep the explants at the gas-medium interface. This method has been successfully used for maintaining three-dimensional development of such cultured soft tissues as skin and neurons and hard tissue as embryonic molars. The chemically defined serum-free medium we used here was previously used for culturing normal neonatal rat palatal mucosa. We also found, after testing various culture media, that this medium with its supplements was the most appropriate for the rat palatal scar tissue organ culture. Additionally, we found that a high concentration of oxygen was essential for maintaining normal relationship between epithelium and connective tissue and for tissue viability. We showed clearly by using this method that the rat palatal scar tissue could be kept in culture in a good condition for 3 days. We also showed that during this period, the histomorphology of the epithelium and that of the connective tissue including α-SMA expressing myofibroblasts were preserved.

Our main concern was to maintain the myofibroblasts marked with α-SMA expression where these cells were surrounded by their original native tissue, including fibers and matrix. Indeed we were able to observe the myofibroblasts in the scar tissue explants in vitro in a manner similar to their appearance in the in vivo situation. Most of the previous investigations regarding the oral mucosa in organ culture have used normal tissues, and many of them have focused on the epithelial component of the mucosa. In contrast, our study is the first to study the premature palatal scar tissue in organ culture and in particular the myofibroblastic component of the connective tissue. Actually, the continuous expression of α-SMA in myofibroblasts of the scar connective tissue as well as in the blood vessels of both scar and normal explants in culture is supportive evidence for the good preservation and viability of the connective tissue component in this model. This evidence might be added to the criteria that we used for evaluating viability of the organ culture model. Although we focused on the myofibroblasts in the connective tissue, it was clear that good preservation and continuous activity of the epithelium occurred, because the epithelial cells proliferated and migrated without epiboly. This could be due to our use of the serum-free medium and the good adherence of the tissues to the filter.

In conclusion, we were able to maintain the rat palatal scar tissue, as well as the normal tissue, could be maintained in culture with good retention of viability and without morphological deterioration over the 3 days of culture. The palatal scar myofibroblasts were highly preserved, and they retained their morphological and biochemical features including α-SMA expression. The rat palatal scar tissue might be a suitable model for studying the accumulation of myofibroblasts, and by using the organ technique it might, therefore, be possible to study the direct effects of various factors or drugs on this tissue.

References


