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EFFECT OF RETINOIC ACID ON CONTRACTION OF COLLAGEN GEL INDUCED BY FIBROBLASTS

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Retinoic acid inhibited the contraction of collagen gel containing fibroblasts. Moreover, in its presence fibroblasts extensions were shorter, fewer collagen fibers were recognized, cell adhesiveness was inhibited concentration-dependently, and microfilaments appeared to be disrupted, resulting in morphological changes including loss of multipolar cell processes due to changes in cytoskeletal linkages.

Residual scarring may lead to undesirable sequelae such as disfigurement, and impaired function. In scar formation, fibroblasts and keratinocytes are known to play important roles in matrix construction and tissue contraction(1). Tissue contraction is considered to be occurred due to actin which is transmitted to neighboring cells or collagen fibers through fibronexus (2). Fibroblasts cultured in collagen gel are similar in bipolar shape to those in vi vo(3-7). Retinoids are a group of natural and synthetic analogs of vitamin A, one of the most biologically potent being retinoic acid. Retinoic acid mediates proliferation and differentiation of normal and abnormal cells by binding to and then activating nuclear retinoid receptor proteins that regulate gene transcription, although the exact mechanisms of its action are not completely understood(8). In addition, retinoic acid affects the adhesiveness of fibroblasts (9). In this study, were examined the causes of inhibition by retinoic acid of contraction of collagen gel by gingival fibroblasts .

MATERIALS AND METHODS

Culture of Gingival Fibroblasts. Human gingival fibroblasts were obtained as described by others(10). The fibroblasts used in this study were obtained from the apically root positioned flap for exposure of an unerupted canine needing eruption guide of a female patient of 10 years old, and amplified in primary culture.

Preparation of Retinoic Acid. Vitamin A acid (tretinoin ;$C_2H_{28}O_2$; MW 300.4) was purchased from Sigma (St. Louis MO.) and the stock solution was serially diluted with ethanol immediately before use. Experiments with retinoic acid were carried out in weak light.

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Preparation of Collagen Gel. Acid soluble type I collagen was purchased from Nitta (0.3% Cellmatrix Type I-A, Niita Gelatine Co., Osaka, Japan). Three dimensional collagen gels were prepared essentially as described by Montesano and Orchi (11). Briefly, sterile instruments and the following solutions used for experiments were placed on ice: (1) 0.3% Cellmatrix Type I-A, (2) 10 times concentrated antibiotic (5000 unit/ml penicillin), antifungal (250mg/ml amphotericin B), and α-MEM without sodium bicarbonate, (3) as a buffer solution for reconstruction, liquid 2.2g sodium bicarbonate and 4.77g HEPES (Wako Co. Japan) were added to 100ml of 0.05N NaOH solution to give a concentration of 20mM HEPES after reconstruction. Seven volumes of 0.3% Cell matrix Type I-A collagen gel at a concentration of 2.1mg/ml were mixed with two volumes of 10 fold concentrated α-MEM containing a concentration of 10^{-5},10^{-6},10^{-7},or 10^{-8}M retinoic acid and one volume of the buffer solution. A pellet of gingival fibroblasts was suspended in the cold collagen mixture (1mg/ml). Aliquots (2ml) of the collagen-cell mixture ($2x10^5$ cells per 2ml collagen mixture) were placed in the 35mm wells of 6-multiwell culture dishes (Sumilon MS 8006R SB Medical Co., Japan) and allowed to gelate for 30 minutes at 37^0C. Then the gels were overlaid with 2ml of medium, and incubated in a high humidity incubator under 5% CO_2 and 95% air.

Measurements of Gel Contraction. The average of the major and minor axes of gel was determined to the nearest 0.01mm from photographs taken every day during the experimental period after gelation.

Effects of Retinoic Acid on Human Gingival Fibroblast Adhesiveness. Adhesion was assayed as described by Lacroix et al.(9). That is, volumes of 4ml of 0.01% trypsin/0.02% EDTA were added the 60mm culture dishes. After agitation at room temperature for 10 minutes, detached and adherent cells were collected from triplicated culture dishes and lyophilized and their contents were measured.

Histological Observations. Collagen fibers in formalin-fixed specimen were identified by Azan staining.(12-13).

Immunofluorescence Staining. The distribution of actin-containing filaments was studied by the indirect immunofluorescence method using primary antibody (mouse monoclonal anti-actin, Amersham England, HP 7 9NA). The fluorescence of isothiocynate was observed after fixation with acetone using a fluorescence microscope (M-F44 Olympus, Tokyo, Japan).

RESULTS

Effect of Retinoic Acid on Contraction of Gel. Collagen gel containing fibroblasts was initially translucent (Fig.1, A), but on contraction it became opaque (Fig.1, B). No contraction occurred in the absence of cells. The effects of various concentrations of retinoic acid on contraction of collagen gel by human gingival fibroblasts are shown in Fig.2. Its inhibitory effect on collagen gel contraction was maximum at a concentration of 10^{-5}M,

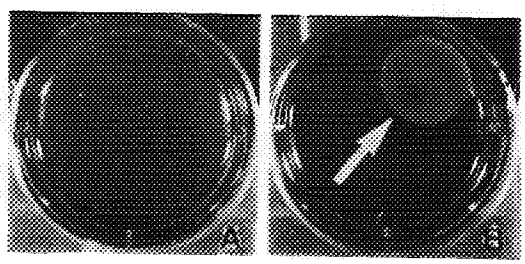

Fig. 1. Contraction of collagen gel containing fibroblasts. Appearances of collagen gel immediately after preparation (A) and after incubation for 7 days (B).

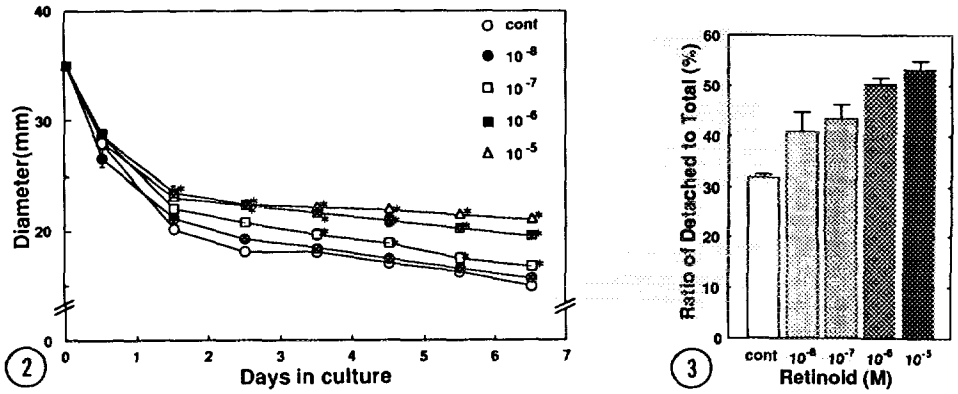

Fig.2. Dose-dependent effect of retinoic acid on contraction of gel containing fibroblasts. Values are means for reduction in size in triplicate dishes. Vertical bars show mean standard deviations(vertical bars cannot be identified because of slight differences), and asterisks indicate values significant lower than those of the control group ($p<0.001$,Student's t-test).

Fig.3. Effect of retinoic acid concentration on the inhibition of cell adhesiveness. Values are means of SD for three determinations.

decreasing the diameter from 35.0mm to 21.7mm at the end of the experiment. The rates of gels contraction were influenced by their contents.

Effect of Retinoic Acid on Adhesiveness. On treatment with retinoic acid, the adhesiveness of cells to plastic culture dishes changed. Figure 3 shows the dose-dependence of the retinoic acid on percentage detachment of fibroblasts. Adhesion decreased with increase the concentration of retinoic acid.

Microscopic Appearance. In the presence of 10^{-5} M retinoic acid, axial processes were strikingly shorter, and fewer collagen fibers were noted. Many collagen fibers were observed in the vicinity of regions of cell extensions, but interconnections of collagen fibers were fewer and poorer in the retinoic acid treatment group (Fig.4, B).

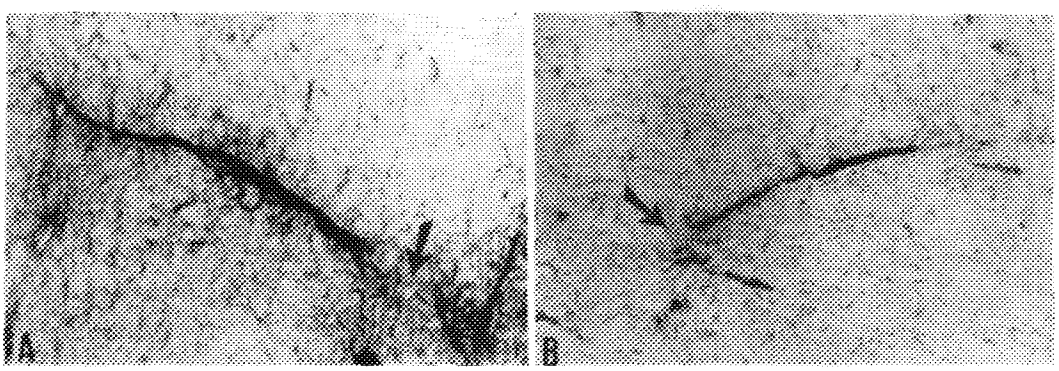

Fig.4. Appearance of human gingival fibroblasts growing on collagen gel. Cells have processes and are attached to each other by collagen fibers (arrows). A. Control. B. Treated with 10^{-5} M retinoic acid. Three days after seeding

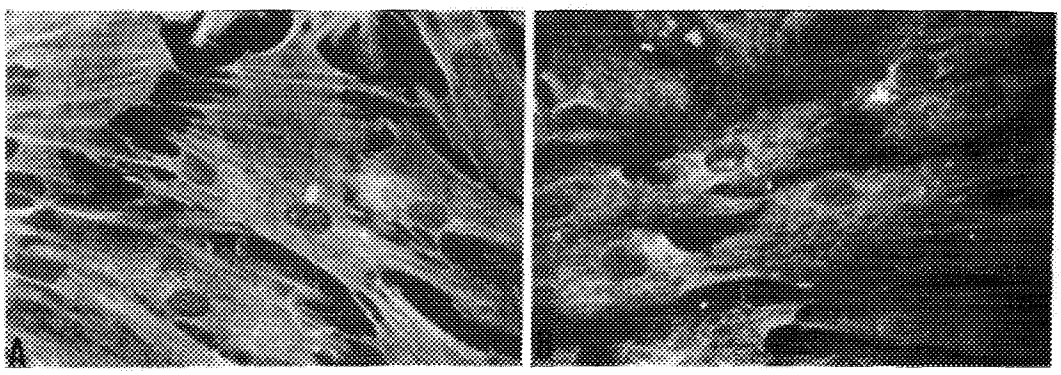

Fig.5. Immunofluorescence staining.A. control,B. retinoic acid,treated.

Cytoskeletal Changes. Treatment of cells with retinoic acid appeared to disrupt microfilaments, resulting in morphological changes including loss of multipolar processes (Fig.5).

DISCUSSION

The contraction of collagen gel containing fibroblasts is similar in many ways to the process that occurs during contraction of healing of wounds, and the contracted gels resemble skin in color and texture. Dermal skin replacement has been reconstructed by collagen gel mixed with fibroblasts, and succeeded in grafting it as a skin-equivalent tissue of full thickness (14-16). Thus, a pharmacological agent that would suppress the contraction of collagen gel could be of a great clinical importance.

In this study, we examined the effect of retinoic acid on the contraction of collagen gel containing human fibroblast, and showed that its contraction decreased dose dependently.

There have been many studies on the effects of retinoic acids on cell growth and differentiation, but most of these studies were not on normal human cells. Several studies have been on monolayer cultures. Studies using animal cells and monolayer cultures may, however, be irrelevant in determining the mechanism of the effect of retinoic acid in humans in vivo.

Contraction of collagen gel containing fibroblasts has been known for a long time, and there are various opinions about its mechanism. One idea is that contraction of the collagen gel results from the cumulative effects of fibroblasts on collagen fibrils (17). Filopodia that make contact with the surrounding collagen gel may be responsible for contraction of the matrix which invariably occurs when fibroblasts are present in collagen gel (18). Fibroblasts within collagen gels must adhere to collagen fibrils, and constitute the fibronexus, which consists of actin, alpha-actinin, vinculin, talin, and integrin with extracellular matrices, for their effect on the fibers. This can be seen by the ability of fibroblasts to attach, spread, and migrate on a variety of extracellular glycoproteins including fibronection, lamimin, vitronectin , and collagen (19-20).

We showed, that retinoic acid treatment induced changes in cell morphology of fibroblasts and their adhesion on the substratum. In fibroblasts, actin in particular is thought to allow the cells to exert a contractile force by means of the fibronexus, and gels containing the cytochalacin, which inhibits actin filament polymerization showed no collagen gel contraction(21). A role of the actin skeleton has been implicated in cellular functions of fibroblasts, including the fibronexus, and adhesiveness. Treatment of fibroblasts with retinoic acid appears to influence their actin skeleton, resulting in inhibition of contraction of collagen gel.

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