REMODELING OF COLLAGEN: AN *IN VITRO* MODEL OF CONNECTIVE TISSUE

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ABSTRACT

An *in vitro* model of connective tissue was reconstituted by introducing fibroblasts into a three-dimensional space of the hydrated collagen lattice. The model was maintained in culture to investigate morphological and biochemical changes in collagen fibrils. Evidence was obtained that the collagen fibrils in the model were stabilized on one hand and were metabolized on the other hand. Collagen fibrils were contracted and were reorganized by the cells in an early stage of culture. Collagen fibrils in the freshly prepared model were easily hydrolyzed by bacterial collagenase. The fibrils became resistant to the enzyme digestion during culture. The acquired resistance seemed to be partly due to the formation of a complex of collagen with polysaccharides secreted by the cells. The amount of collagen in the model decreased gradually during culture and a hydroxyproline-containing substance or substances accumulated in the medium, indicating that fibroblasts catabolized the collagen fibrils. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis demonstrated that the collagen was degraded to a peptide or peptides with molecular weights less than 20 K. The electron microscopical examinations of the model revealed some bizarre bodies with a striated banding pattern, which resembled the zebra-bodies reported *in vivo*. The origin of this striated structure is discussed.

Cells embedded three-dimensionally in a matrix of collagen lattices have been known to behave as they do *in vivo*. Primary mammary tumor epithelial cells in collagen gels sustained growth and produced duct-like structures (14). A similar *in vitro* system has been used successfully for the growth of mouse vaginal epithelial cells (7). The cells described above do not proliferate in the conventional monolayer culture. Fibroblasts in

the collagen gel condensed a collagen lattice to a tissue which resembles dermis in color and texture (1, 4).

These studies strongly support an attempt to develop *in vitro* models of normal tissue and organ that mimic the function and structure of the *in vivo* counterparts. The models can be reconstituted by introducing constituent cells of a tissue into the three-dimensional space of extracellular matrices as originally developed by Elsdale and Bard (3). These models appear to be useful for studying metabolism of extracellular substances regulated by resident cells, which remains poorly understood.

In the present study, a model of connective tissue was reconstituted, where fibroblasts were maintained three-dimensionally in hydrated col-

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Abbreviations: DAB, 3,3'-diaminobenzidine-HCl; FITC, fluorescein isothiocyanate; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethansulfonic acid; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane

lagen lattices to investigate metabolic fate of collagen fibrils in the model.

MATERIALS AND METHODS

Chemicals and culture materials were purchased from the following sources: HEPES and 3,3'-diaminobenzidine-HCl (DAB) from Dojin Chemical Institute; fluorescein isothiocyanate (FITC) from Becton-Dickinson; fetal calf serum (FCS) from Commonwealth Serum Laboratory; antibiotics (streptomycin and penicillin) from Meiji Seika Kaisha; collagenase (form III, *Clostridium histolyticum*, nonspecific protease-free, EC 3.4.24.3) from Advance Biofactures; hyaluronidase (beef testes and *Streptomyces hyalurolyticus*, EC 3.2.1.35), chondroitin ABC lyase (EC 4.2.2.4) and chondroitin AC lyase (EC 4.2.2.5) from Seikagaku Kogyo; biotinylated lectins (wheat germ agglutinin, *Ricinus communis* agglutinin, Concanavalin A, *Dolichos biflorus* agglutinin, peanut agglutinin, soybean agglutinin) and horseradish peroxidase-avidin D from Vector Laboratories; Eagle's minimum essential medium (MEM) and Dulbecco's modified Eagle's MEM (DEM) from GIBCO; Falcon culture dishes from Becton Dickinson Labware; and Millipore filters (type HAWP and HVLP) from Japan Millipore. All other chemicals were reagent grade and purchased from Wako Pure Chemical Industries or Nakarai Chemicals.

Preparation of Collagen

Collagen was prepared from calf dermis and characterized as described previously (15). FITC-labelled collagen was prepared as follows. One gram of powder of collagen was solubilized in 250 ml of 0.01 M CH_3COOH . An equal volume of 0.5 M NaHCO_3 - Na_2CO_3 buffer (pH 9.5) was added to the collagen solution. Forty milligrams of FITC dissolved in 5 ml of acetone was mixed with 10 ml of 0.5 M NaHCO_3 - Na_2CO_3 buffer. The collagen solution was mixed with the FITC solution and the mixture was stirred for 3 h at 25°C. The FITC-labelled collagen was precipitated by adding 100 g of NaCl and standing overnight at 4°C. The precipitate was washed extensively with 20% NaCl and dissolved in redistilled water (pH 3). The collagen solution was dialyzed against redistilled water (pH 3) and sterilized by passing through 0.8 μm and 0.45 μm Millipore filters (HAWP) in this sequence. Fluorescence of the collagen solution was measured with a Hitachi fluorospectrometer (model 650-60) at 495 nm of excitation and at

520 nm of emission.

Cell Culture

Human fibroblasts were obtained from explants of the normal dermis of a 1-year-old female (15). Using standard techniques (10), fibroblasts were grown and maintained in 75-cm² plastic culture flasks in DEM medium containing 10% FCS, 10 mM NaHCO_3 , 20 mM HEPES, 100 units/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin in a moist atmosphere of 5% CO_2 /95% air at 37°C. Cells between 5 and 14 doublings were used in the experiments.

Reconstitution of a Model of Dermal Connective Tissue

Fibroblasts were embedded three-dimensionally in hydrated collagen lattices by a modified procedure of Elsdale and Bard (3). The following stock solutions were prepared and kept below 4°C: a 0.5% (w/v) collagen solution (2.8 $\times$ concentrated); 10 $\times$ Eagle's MEM; 1 M HEPES (50 $\times$); 1 M NaHCO_3 (100 $\times$); 205 mM glutamine (100 $\times$), 100 mM sodium pyruvate (100 $\times$); a 100 $\times$ antibiotics mixture (10 mg/ml streptomycin and 10,000 units/ml penicillin); and FCS (10 $\times$). These solutions were mixed with glass-redistilled water to give the each solution one strength and the pH was adjusted to 7.4 by adding 1 N NaOH. Fibroblasts were suspended with this collagen-containing medium and poured into plastic dishes. The dishes were transferred into an incubator at 37°C. Collagen was gelled within 10 min and the cells were trapped three-dimensionally in the gel. If not specified otherwise, 2 ml of the model containing 0.18% collagen and 2.5×10^5 fibroblasts was prepared in a 35-mm dish. For long-term culture, the model was overlaid with 1.5 ml of a culture medium and the medium was changed twice a week.

Collagenase Digestion

The culture medium (1.5 ml) was removed from the model and replaced with a fresh culture medium containing 100 units of bacterial collagenase. The model was incubated at 37°C for varied lengths of time. The model was transferred to a plastic tube together with the medium and centrifuged at 11,000 g for 30 min. The amount of collagen hydrolyzed by the enzyme was determined by measuring hydroxyproline in the supernatant (2). In the experiment shown in Fig. 4, the model was pretreated with a glycos-

aminoglycanase (testicular hyaluronidase, *Streptomyces* hyaluronidase, chondroitin AC lyase or chondroitin ABC lyase), and then digested with collagenase. The enzyme-containing medium was sterilized by filtrating through a Millipore filter (type HVLP) prior to use.

Stain of Collagen with Lectins

Dishes with hydrated collagen lattices were washed well with Hanks' solution and were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) at 4°C overnight. After washing with 10 mM phosphate-buffered saline (PBS) (pH 7.2) containing 0.1 M lysine, the collagen gel was dehydrated with a graded series of ethanol and was embedded in paraffin. The sections (1 μ m thick) were incubated in PBS-lysine for 30 min at room temperature and then with 50 μ g/ml biotinylated lectins for 60 min. The sections were rinsed well in PBS-lysine to remove free lectins and were incubated in PBS containing a 50 μ g/ml avidin-horseradish peroxidase complex for 30 min. The sections were washed well, processed for color development by incubating for 3 min with a DAB-H₂O₂ solution (0.25 mg/ml DAB and 0.005% H₂O₂ in 50 mM Tris-HCl buffer, pH 7.6), and dehydrated. The extent of color development of the sections was assayed by observing through a light microscope after they were mounted in balsum.

Histological Stain

The cultured model of connective tissue was

fixed in 10% phosphate-buffered formalin and was processed with an ordinary histological procedure. The model was stained by Masson's trichrome stain (13).

Electron Microscopic Observations

The collagen gel with fibroblasts was washed with Hanks' solution and was fixed with 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) containing 1% tannic acid for 2 h at 4°C. The fixed gel was washed with PBS and was post-fixed with 1% OsO₄ in 0.1 M phosphate buffer (pH 7.4) for 2 h at 4°C, followed by dehydration in a series of graded alcohol and by embedding in epoxy resin. The ultrathin sections were double-stained with uranyl acetate and lead acetate, and were examined with a Hitachi HU-12A transmission electron microscope (TEM).

SDS-Polyacrylamide Gel Electrophoresis

Degradation products of collagen in the model were analyzed by SDS-polyacrylamide gel electrophoresis. The model of connective tissue was reconstituted as described above except that FITC-labelled collagen was used in place of collagen. An aliquot of the medium where the model had been cultured was subjected to SDS-polyacrylamide gel electrophoresis (8). Fluorescence was detected by placing the dry gel on an UV lamp.

Fig. 1 Microscopic appearance of a model of connective tissue. Half million fibroblasts were embedded in 2 ml of a 0.18% collagen gel and were cultured for 9 days. A: The model was stained with Masson's trichrome stain. Condensation of collagen fibrils is evident adjacent to fibroblasts. $\times 240$. B: A transmission electron microscopic observation. Fibroblasts in the model reorganized collagen fibrils, resulting in a ordered arrangement of the fibrils. $\times 3,000$

RESULTS

Rearrangement of Collagen Fibrils

When the model of connective tissue was cultured, collagen lattices thinned to less than one-third of its original thickness, squeezing fluid out of the meshes of collagen (1), as a result of contraction of collagen fibrils by fibroblasts (Fig. 1A). Electron microscopical observations demonstrated that collagen fibrils were condensed near fibroblasts and were oriented in parallel with the cell surface (Fig. 1B).

Stabilization of Collagen Fibrils against Collagenase Digestion

Hydrated collagen lattices including fibroblasts were cultured for up to 40 days and were digested with pure bacterial collagenase (Fig. 2). The freshly prepared lattice that contained 3.6 mg of collagen was completely solubilized within 32 h by 100 units of the enzyme. As the model was cultured, the lattice became resistant against the attack of the enzyme. About 35% of the collagen in a model cultured for 20 days remained undigested and 80% was not hydrolyzed in a 40-day-old model. Collagen lattices incubated for up to 40 days without fibroblasts did not acquire the resistance to the attack of collagenase. Fig. 2 demonstrates the other aspect of the interaction of fibroblasts with collagen fibrils. The amount of collagen that remained in the model decreased with the time of culture (see time 0 in Fig. 2). This phenomenon is detailed below. The extent of the resistance was proportional to the number of fibroblasts embedded in the model (Fig. 3).

To gain an insight into the mechanism of acquirement of the collagenase-resistance in collagen fibrils, the possibility was examined that the fibril forms a complex with proteoglycans produced by fibroblasts. In the experiment of collagenase digestion, the model was treated with one of several glycosaminoglycanases (Fig. 4). The collagenase-resistance was weakened significantly by the pretreatment with chondroitin ABC lyase and to some extent by chondroitin AC lyase or testicular hyaluronidase. Bacterial hyaluronidase did not affect the resistance. The possibility mentioned above was also suggested by the other experiment. The reactivity of the fibroblast-populated collagen fibrils with lectins was different from that of the fibrils incubated without the cells (Table 1). *Dolichos biflorus* agglutinin and peanut agglutinin became bound to the collagen fibrils which were incubated with fibroblasts for 20 days. Soybean agglutinin also

Fig. 2 Digestion of the connective tissue model with bacterial collagenase. The model was reconstituted by embedding 5×10^5 fibroblasts in 2 ml of 0.18% hydrated collagen lattices. After incubating for varied lengths of time, the model was digested with pure bacterial collagenase as detailed in the text. At the time indicated in the figure, the amount of undigested collagen (fibrillar collagen) was determined by measuring hydroxyproline (HYP) in the precipitate obtained by centrifugation of the digested model after HCl-hydrolysis at 110°C for 24 h. The collagen lattice without the cells was hydrolyzed by the enzyme at the same rate as the freshly prepared model (day 0). Each point represents the mean value of two determinations and the bar shows their range. $\circ$ — $\circ$, the freshly prepared model; $\bullet$ — $\bullet$, the model cultured for 20 days; $\blacksquare$ — $\blacksquare$, the model cultured for 40 days

became reactive to the fibrils, although the extent was not strong as compared with the two former lectins.

Solubilization of Collagen Fibrils in the Model

As suggested in Fig. 2 and Fig. 3, some of the collagen fibrils in the model were solubilized into the medium and were removed from the dish upon medium change. The solubilization of collagen fibrils was investigated quantitatively during a period of culture of the model.

Collagen that participated in a fibril formation was sedimented by centrifugation at $11,000\text{ g}$ for 30 min. About 25% of collagen in the freshly prepared hydrated collagen lattice was not in a fibril

Fig. 3 Collagenase digestion of models with different numbers of fibroblasts. Connective tissue models were prepared by embedding varied numbers of cells in 2 ml of 0.18% collagen lattices. They were cultured for 20 days and were digested with bacterial collagenase as described in the text. The amount of a fibrillar form of collagen was determined as described in Fig. 2. Each point represents the mean value of two determinations and their range is shown by vertical lines. Open columns represent the models without the enzyme digestion and closed columns the models treated with the enzyme.

Table 1 Reactivity of Lectins with Collagen Fibrils Incubated with or without Fibroblasts

Lectins tested	Days in culture			
	Day 0		Day 20	
	Without cells	With cells	Without cells	With cells
Wheat germ agglutinin	++	++	++	++
<i>Ricinus communis</i> agglutinin	++	++	++	++
Concanavalin A	+	+	+	+
<i>Dolichos biflorus</i> agglutinin	—	—	—	++
Peanut agglutinin	—	—	—	++
Soybean agglutinin	—	—	—	+

The connective tissue models were prepared by introducing 5×10^5 fibroblasts in 2 ml of a 0.18% collagen gel. The reactivity of collagen fibrils with lectins was assayed as described in the text and was expressed as —, +, or ++: —, negative in the color development; +, positive; ++, highly positive.

form but in a soluble form (Fig. 5). The soluble collagen in the model decreased gradually during a period of culture because it was removed upon medium change. The amount of collagen in a fibril form also decreased gradually during the same period only when fibroblasts were present in the lattice, suggesting that some of the collagen fibrils are metabolized by the cells.

The amount of collagen released into medium was determined during a period of culture of the model by measuring hydroxyproline in the me-

dium. A hydroxyproline-containing substance or substances were released from the model and increased in the amount in proportion to the length of culture period. The amount of the substance that accumulated in the culture medium was about $30 \mu\text{g}$ as hydroxyproline when the model with 5×10^5 cells was cultured for 20 days. Process of the release of collagen was monitored in the model constructed with FITC-labelled collagen (Fig. 6). Accumulation of the FITC-containing substance in the medium was apparent

Fig. 4 Effect of glycosaminoglycanases on the extent of digestion of collagen lattices with collagenase. The models contained one million fibroblasts in 2 ml of a 0.18% collagen gel and were cultured for 30 days. Models were pretreated with one of the glycosaminoglycanases indicated in the figure for 24 h. The concentration of enzymes added was as follows: chondroitin AC lyase (CHase AC), 0.2 units; chondroitin ABC lyase (CHase ABC), 0.2 units; *Streptomyces* hyaluronidase (HYAase(S)) 80 units; and testicular hyaluronidase (HYAase(T)) 100 units. Then, 100 units of bacterial collagenase was added to the models and they were digested for additional 32 h. The extent of digestion was determined and was expressed as in Fig. 3. Each value represents the average of two determinations and the bar shows their range. The symbols of + and - indicate the presence and absence of cells or the enzyme concerned during incubation, respectively.

and was dependent on both the length of a culture period and the number of the cells embedded.

Fibroblasts were embedded in the FITC-labelled collagen lattices. To obtain degradation products of collagen fibrils, the medium was collected on the third and 7th day of culture and analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 7). Two bands were detectable by fluorescence in the model with cells: *i.e.* the γ -chain of a collagen molecule and a highly degraded form (arrow). The molecular weight of the latter was less than 20 K.

Appearance of Zebra-bodies

To see morphological changes in the collagen fibrils, the model was surveyed by an electron microscope. Only when the fibroblasts were populated in the model, some fiber-like structures with cross-striations were detectable (Fig. 8A, arrows). They were in a spindle form. Ends of these fibers were tapered. These bizarre structures were tentatively called 'zebra-bodies' because of resemblance in their appearance to 'zebra-bodies (Luse's bodies)' described by Luse (6, 9). The body had a dense cross-banding of 70

Fig. 5 Decrease in the amount of collagen fibrils in the model during culture. Half million fibroblasts were cultured in 2 ml of a 0.18% collagen gel for up to 40 days. As a control experiment, collagen gels without the cell were prepared and were processed similarly as in the experimentals. Models were centrifuged at 11,000 g for 30 min to separate a fibrillar form of collagen from a soluble form. The amount of collagen was determined by measuring hydroxyproline (HYP) after HCl-hydrolysis at 110°C for 24 h. Each value represents the average of two determinations, the range of which is shown by vertical lines. Symbols of — and + indicate the absence and presence of the cells in the collagen gels. Open columns represent a soluble form of collagen and closed columns a fibrillar form.

to 80 nm. Size of the striated structures was variable and there was no correlation between the size of the bodies and length of a culture period. Usually large bodies were not in a typical spindle form and contained decomposed thin filaments (Fig. 8B, arrows). In other cases, the zebra-body was found in the cell (Fig. 8C, arrows). The zebra-bodies were not discernible in the early period of culture. They were observed after around 7 days of culture with a relatively higher frequency.

DISCUSSION

In the present study, a model of dermal connective tissue was reconstituted by embedding human dermal fibroblasts in hydrated collagen lattices and remodeling of collagen fibrils by fibroblasts was investigated morphologically and biochemically.

A morphological study on collagen fibrils has

Fig. 6 Release of collagen into the culture medium from the connective tissue model. The model was prepared by embedding varied numbers of fibroblasts in a 0.18% FITC-labelled collagen gel. One and half milliliters of the culture medium was overlaid on the model. The culture medium was changed at the days indicated and was saved for the determination of fluorescence. The results were expressed as the ratio (%) of collagen released into the medium to collagen added to the culture dish. Each point represents the average of two determinations, which were within 5% of the average. ●—●, without cells; ▲—▲, 5×10^5 cells; ■—■, 10^6 cells

been reported recently by Grinnell and Lamke (4). They noticed that collagen in the fibroblast-populated collagen lattices was reorganized by the cell and aligned in the plane of cell spreading. Based on their finding that there was very little collagen degradation or synthesis, they claimed that the change in the collagen lattice represents a true reorganization. Our results show that some of the collagen in the lattice degrade gradually during a culture period, which suggests that remodeling of collagen lattices involves selection of collagen fibrils by the cells and the fibrils which remains undigested participate in the reorganization.

Recently Nusgens *et al.* (12) described that fibroblasts in a tissue equivalent matrix expressed a relatively higher collagenolytic activity as compared with the cells in a conventional monolayer culture. They did not present any actual data on this and only stated the result of their observation. We performed quantitative experiments on the degradation of collagen fibrils in the model and clearly demonstrated that the fibroblasts degrade the fibrils to small peptides. At present, it is not clear whether collagenase is involved in the process of collagen

Fig. 7 The SDS-polyacrylamide gel electrophoretic pattern of collagen released into the culture medium. The model was prepared by embedding one million fibroblasts in 2 ml of a 0.18% FITC-labelled collagen gel and was cultured for the days indicated with 1.5 ml of an overlaid medium. An aliquot of the medium was subjected to SDS (0.1%) polyacrylamide gel electrophoresis (10% acrylamide). The photograph was taken by placing the dried gel over an UV lamp. C represents the FITC-labelled collagen as a marker. Symbols of + and - indicate the presence and absence of cells in the model. α , β and γ represent the corresponding chain of a collagen molecule. The arrow indicates the position of a degraded collagen(s). Molecular weights are shown on the left.

degradation observed in the model, because degradation products known as TC^A and TC^B (5) were not detected in the protein banding pattern in SDS-polyacrylamide gel electrophoresis (Fig. 7).

We observed some bizarre fragments named zebra-bodies. The similar cross-banded structures have been described in the nervous system (11), in various tumors of neural origin (9) and in the stroma of squamous cell carcinoma and malignant melanomas of the skin (6). In the last case, no cross-banded structure was found where collagen fibrils did not show degradative changes, suggesting a possibility that compensatory production of tropocollagen in the abnormal stroma may contribute to production of the zebra-bodies (6). All the zebra bodies reported hitherto were observed *in vivo*. The similar cross-striated structure was found *in vitro* in the present study, where fibroblasts and collagen fibrils were main constituents. At an early period of the culture the structures were hardly observed. They were discernible after one week of culture. We postulate that the zebra-bodies might be an intermediate of degradation of collagen fibrils by fibroblasts, which would be finally phagocytosed

by the cells for the following reasons. Firstly, it has been reported that the fibroblasts in the model show quite low activity of collagen biosynthesis (12), which might rule out the possibility cited above that zebra-bodies are formed as a result of compensatory production of tropocollagen (6). Secondly, the zebra-bodies took on a degenerative appearance. Usually they showed a symmetrical spindle form. However, the symmetry was disturbed and thin filaments were exposed in large zebra-bodies (Fig. 8B). Finally, the zebra-bodies, though rarely, were detected within the cell, suggesting that fibroblasts might phagocytose them. The chemical nature of the body remains to be resolved and should be critical for the identification of the body. There remains a possibility that the zebra-bodies are produced not by the fibroblasts, but by macrophages that might contaminate in this model. It may be possible that a small number of macrophages contaminated in the primary culture of fibroblasts. However, this possibility does not seem to be plausible, because the model was prepared by fibroblasts with a population doubling level of 11. The number of macrophages must have been reduced to a negligible level during

Fig. 8 Zebra-bodies which appeared in the model during culture. The model of connective tissue was prepared by embedding 5×10^5 fibroblasts in 2 ml of a 0.18% collagen gel. A: Relatively small zebra-bodies in a spindle form (arrows). The photograph was taken after culturing the model for 10 days. $\times 18,000$. B: Large zebra-bodies. The specimen for the electron microscopy was obtained on the 10th day of culture. Note that the zebra-bodies were not in a typical spindle form, but a part of them contained decomposed thin filaments (arrows). $\times 21,000$. C: Zebra bodies in the cell (arrows). The specimen was obtained on the 20th day of culture. $\times 10,000$

several passages of subcultivation, for the following two reasons. Firstly, macrophages are known to be unable to proliferate *in vitro*. Secondly, macrophages remain attached to plastic dishes, when cells are exposed for a short time (less than 5 min at 25°C) to a trypsin-EDTA solution for subcultures of fibroblasts, and are removed from a population of fibroblasts.

In the connective tissue model two events, which appear to be contradictory to each other, are progressing simultaneously: degradation and stabilization of the collagen fibrils. Both events are fibroblasts-mediated changes. According to the results of the present study, we put forward a possible process of extracellular metabolism of collagen. As shown in Fig. 5, not all the mono-

meric collagens added to the model participate in the formation of fibrils which are insoluble in the culture medium. Twenty five percent of the added collagen remains soluble, which is thought to be the first portion to be degraded by the cells. During culture, some fibrils are degraded by the cells and others should become complexed with substances secreted by the cells, most probably sulfated glycosaminoglycans, and they are protected against attack of lytic enzymes. It is not known whether or not this process takes place *in vivo*. However, the fibroblast-populated model of connective tissue would be a valuable experimental system to analyse the remodeling and metabolism of collagen fibrils. The analysis with this model will provide useful information to estimate the actual events occurring *in vivo*.

This work was supported in part by a grant-in-aid for special project research from the Ministry of Education, Science and Culture, Japan (No. 59105003), and by a grant for Special Research Projects of Kitasato University School of Medicine (No. 83502).

Received for publication 21 May 1985; and in revised form 11 July 1985

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