Evaluation of Collagen Gel Microstructure by Scanning Electron Microscopy

A. G. Pogorelov[1,2] and I. I. Selezneva[1,2]

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We performed qualitative comparison of freeze drying and chemical drying as methods of preparing 3D wet specimens for scanning electron microscopy. Human fibroblasts immobilized in collagen gel were used as a model system. Specimens fixed with glutaraldehyde were frozen in liquid nitrogen and freeze-dried at low temperature in high vacuum. In parallel experiments, glutaraldehyde-fixed samples were dehydrated in ascending ethanol solutions, absolute ethanol, and 100% hexamethyldisilazane and then dried at room temperature. Scanning electron microscopy microphotographs of collagen fibers and cells were characterized by high resolution and the absence of collapsed or deformed structures even at high magnification (×50,000) for both chemical drying and high-vacuum freeze drying. However, high-vacuum freeze drying is superior to chemical drying for the investigation of the internal space of 3D scaffolds, because sample fracture can be prepared directly in liquid nitrogen. These techniques are a part of the sample preparation process for scanning electron microscopy and can also be used for studies of cell adhesion, morphology, and arrangement in wet specimens (3D gels and flexible tissue engineering scaffolds).

Key Words: *collagen gel; fibroblasts; scanning electron microscopy; freeze drying*

Cell culturing in 3D hydrogel scaffolds creates models approximating *in vivo* conditions. Collagen fibers form a network affecting cell flattening and, hence, modulating their proliferative activity and differentiation. Culturing of fibroblasts in a collagen gel leads to its shrinkage and results in the formation of a compact disk contracting with time [2]. Contraction of the collagen gel by immobilized cells is used as a model of tissue formation and functioning [1]. This approach is also used in reconstructive medicine where gels are used as tissue equivalents.

Porous 3D structure of collagen gels is the basis of modern soft tissue engineering. Collagen sponges containing hydroxyapatite and calcium orthophosphate are used for the creation of implants intended for reconstruction or equivalent replacement of the bone tissue. Problems of cell adhesion, flattening, interaction with other cells, migration into the 3D lattice, proliferation, and differentiation in the required directions are to be solved during creation of this class of materials. Scanning electron microscopy (SEM) is the most efficient method of direct visualization of spatial organization of the object surface [3,5]. However, bioorganic materials (gels) with low dry residue index usually undergo thermal degradation under electron beam at high magnification. This leads to focus instability and impairs image quality.

In the present study, a method of collagen material preparation for structural studies on the basis ultrarapid cryofixation in liquid nitrogen followed by low-temperature high-vacuum dehydration was developed. We studied structural organization of collagen hydrogels containing fibroblasts isolated from musculocutaneous tissue of human fetuses. After fixation in glutaraldehyde, the samples were either dehydrated routinely by passing through ascending concentrations of ethanol

[1]Institute of Theoretical and Experimental Biophysics, Russian Academy of Sciences; [2]Pushchino State University, Russia. ***Address for correspondence:*** agpogorelov@rambler.ru. A. G. Pogorelov

and hexamethyldisilazane (HMDS) or subjected to low-temperature high-vacuum dehydration. The structure of collagen gels was studied and the results obtained by using the method developed by us and standard methods of sample processing for SEM were compared.

MATERIALS AND METHODS

Type I collagen was extracted from caudal tendons of Wistar rats with 0.5 M acetic acid. After neutralization (pH 7.0) of the extract with ammonia aqueous solution, collagen fibers were precipitated by adding 20 vol% ethanol. After 30-min centrifugation at 5,000g at 5°C, the collagen sediment was dissolved in 0.1 M acetic acid. Its concentration was measured by the weight of dry residue. Collagen stock solution with a concentration of 6 mg/ml was used in the experiments. For gel preparation, the solution of type I collagen was mixed with culture medium, pH was adjusted to neutral with 1 M NaOH.

Cells isolated from the musculocutaneous tissue of human embryos (gestation weeks 6-8) were used. Human embryonic fibroblasts were obtained by enzyme disaggregation of embryonic tissues using the following procedure: aseptically isolated tissues were washed with DMEM, minced to 0.5-mm fragments, and treated with 0.25% trypsin at 37°C for 15-20 min. The isolated cells were centrifuged at 1500 rpm for 2 min and cultured in DMEM supplemented with 10% FCS. Fractionation of the primary mixed culture was carried out using differences in adhesion time for different cell types. Passage 11 cell culture was used ($CD133^-$, $CD117^-$, $CD45^-$, $CD90^+$, $CD54^-$, $CD62L^-$, $CD62P^-$, $CD9^+$, $CD34^-$, $CD31^-$, $CD71^-$, $CD20^-$, $CD157^-$, $CD106^+$, $CD62E^+$).

For cell immobilization, the cell suspension was mixed with collagen solution and the mixture was heated at 37°C. One cubic centimeter of the gel contained 1 mg protein and 100,000 cells. Further culturing was performed in DMEM/medium 199 (1:1) containing 10% FCS (HyClone) and 100 U/ml penicillin/streptomycin at 5% CO_2 for 1 week. After completion of gel contraction, the samples were washed with 0.1 M PBS (pH 7.4) and fixed in 2.5% glutaraldehyde on the same buffer for 12 h at 5°C.

Material for SEM was prepared by two methods. In the first case, gel samples after fixation were washed with PBS and dehydrated at 4°C in ascending con-

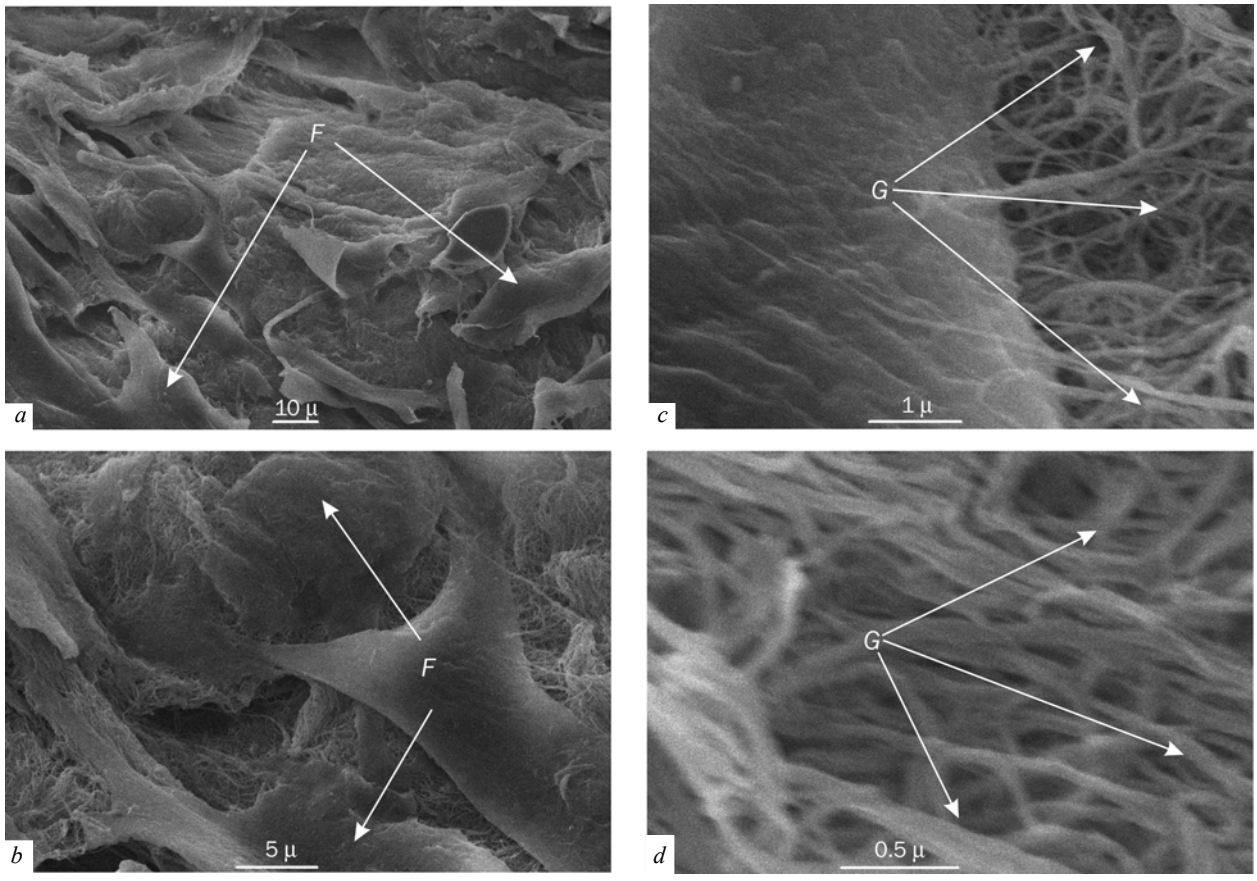

Fig. 1. SEM of surface relief of collagen gel prepared by water replacement with HMDC followed by removal of the solvent by air-drying (secondary electron mode). Here and on Fig. 2: *a-d*: gel surface at different magnifications. *F*: fibroblasts; *G*: fine gel structure.

centrations of aqueous ethanol solutions (50, 75, 80, and 90%) and absolute ethanol (2×5 min for each concentration). Then the samples were plunged into HMDS for 30 min for removing ethanol and dried on air [6]. In the second case, ultrarapid cryofixation of the sample was used [4]. The gel was thoroughly washed off the fixative with distilled water, the sample was snap frozen in liquid nitrogen (-196°C) and broken with cold forceps. A fragment of frozen gel was transferred into a chamber of Micro BA3 (Balzers) apparatus for low-temperature (-100°C) high-vacuum (10^{-4} Pa) dehydration. The samples prepared by these two methods were mounted onto an object holder of an electron microscope using conducting glue. Platinum coating (20 nm) was applied on the specimen using a JFC 1600 coater (JEOL) for metal evaporation in argon atmosphere. The relief of specimen surface was studied under a JSM-6390A scanning electron microscope JEOL at accelerating voltage of 10 kV operated in secondary electron mode.

RESULTS

The difficulty of specimen preparation for SEM is determined by high water content (~80%), which requires specimen dehydration. The procedure of drying is the main cause of violation of the fine structure of the specimen. There are two main causes of artifacts: relief changes due to surface tension and precipitation of substances from the bathing solutions on the specimen surface. Therefore, simple drying of the sample on air after chemical fixation in water solution of the fixative (*e.g.* glutaraldehyde) is impossible. Processing of gels with low dry residue content (fractions of percent) requires particular care.

To avoid mechanical deformation of structures on the surface of the examined specimens and its impurity, classical histological methods are used: the specimens are passed through a battery of aqueous solutions of ethanol with increasing concentrations. In SEM protocol, absolute ethanol at the final stage is replaced with HMDS [6]; this substance is then removed by the method of critical point drying as described previously [3] (Fig. 1).

An alternative approach is based on cryogenic methods of electron microscopy [3,4]. This approach, if used immediately after chemical fixation, does not imply water replacement with another solvent. The specimen is subjected to ultrarapid physical fixation in a cryoagent, *e.g.* liquid nitrogen, followed by

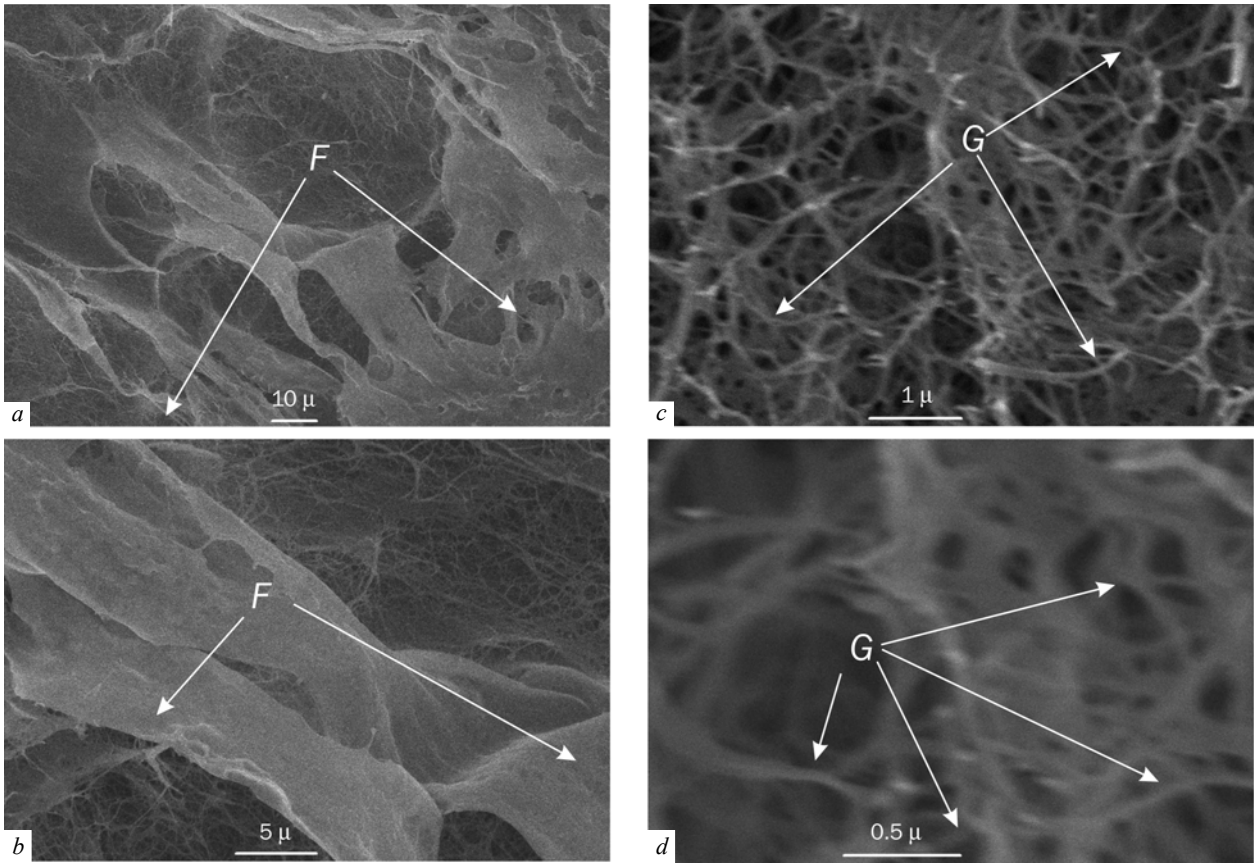

Fig. 2. SEM of surface relief of collagen gel prepared by freeze–fracture method followed by low-temperature vacuum dehydration.

vacuum lyophilization. The frozen water sublimates from the solid state into gas without passing the liquid state. This allows avoiding the effects of surface tension and damage to the fine structure of the specimen under the action of the solvents replacing water. The specimen should be washed with water before freezing; otherwise a powder layer consisting of substances dissolved in the fixative appears on its surface. This coat masks the relief and structure of the specimen surface.

Comparison of microphotographs of collagen gel samples prepared through replacing water with HMDC followed air-drying (Fig. 1) and the same samples processed by low-temperature vacuum dehydration (Fig. 2) showed that both methods allow visualization of collagen fibers and immobilized cells.

Both chemical drying and high-vacuum freezing-drying yielded satisfactory morphological resolution at the level of nanostructures and ensured the absence of collapse and deformations even at high resolutions (up to ×50,000). Thus, it seems useful to employ both methods of specimen processing for SEM examination of 3D biological samples on the basis of collagen gels. In each variant, availability of the corresponding equipment in the limiting factor. The appreciable advantage of the cryogenic approach consists in the possibility of obtaining and studying the surface passing through internal gel layers, which can be achieved by fracture of the sample frozen in liquid nitrogen (freeze–fracture).

Qualitative comparison of chemical and sublimation methods of preparing 3D wet specimens for scanning electron microscopy showed that both methods under proper conditions allow examination of the microstructure of collagen scaffold and morphology of cells immobilized in its volume up to a magnification ×50,000. The proposed method of sublimation of frozen samples is less laborious and provides more possibilities for the study of internal volume of 3D samples by using freeze-fracture technique. This technique as a part of specimen processing for SEM can be used for the study of morphology and organization of cells in 3D gels and scaffolds used for tissue reconstruction.

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