A Novel Method for Efficient Collection of Normal Mesothelial Cells In Vivo

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Summary Asbestos-induced mesothelioma is a challenging social problem in many countries, and oxidative stress via iron is closely associated with its carcinogenesis. Mesothelioma is thought to originate from the mesothelial cells that cover the somatic cavity such as pleural, pericardial and peritoneal cavities. They are single layered and so flat that it is extremely difficult to obtain pure mesothelial cells as control samples from experimental animals. Here we describe a novel method to collect mesothelial cells from animals by the use of simple equipments. Surface of the most organs including lung, spleen and liver are covered with a single layer of mesothelial cells. Scraping the surface of those organs with razor blades after snap-freeze in liquid nitrogen satisfactorily confers almost pure population of mesothelial cells. This simple method would be helpful for obtaining mesothelial control samples from animals to elucidate the molecular mechanisms of a variety of mesothelial pathology.

Key Words: mesothelium, mesothelioma, podoplanin, western blot analysis, immunohistochemistry

Introduction

Mesothelial cells form a monolayer that lines the body’s serous cavities and internal organs. The primary function of this layer, termed the mesothelium, is to provide a slippery, non-adhesive and protective surface. In addition to that, mesothelial cells are also involved in a variety of biological functions such as transport of fluid or cells across the serosal cavities, inflammation and tissue repair [1, 2]. Over the last few years, there is a dramatic increase in the number of studies related to the asbestos-induced mesothelioma, as a result of a steady increase in the number of asbestos-induced mesothelioma patients [3]. Oxidative stress via iron has been closely associated with its carcinogenesis [4–6]. Nevertheless, due to the lack of an effective method to collect control mesothelial cells from fresh tissues, efforts in the study of mesothelioma are impeded to some extent; in the absence of appropriate controls, screening experiments such as expression microarray analyses may miss important findings. Here, we describe a novel strategy to collect mesothelial cells from the surface of fresh organs. This technique would be useful for the researchers who intend to obtain pure mesothelial cell population for biochemical and molecular analyses.

Materials and Methods

Collection of mesothelial cells

The animal experiment committee of Nagoya University Graduate School of Medicine approved this experiment. Eight-week-old Brown-Norway rats (Charles River Japan, Yokohama, Japan) were dissected. Fresh rat organs were taken and snap-frozen in liquid nitrogen. The frozen organs were then taken out from liquid nitrogen and put on dry ice. Apparent frost formed on the organ due to temperature change was carefully removed with a thin razor blade. The same razor blade, which was cooled on the dry ice, was held...
at a perpendicular angle to the surface of organ and used to scrape the single layer of mesothelial cells from the surface in a unidirectional manner from far to near (Fig. 1A). The small amounts of frozen crusts on the tip of the blade were carefully collected to sample tubes. It was kept at −80°C until analytical use.

Cell cultures
Rat peritoneal mesothelial cells (RPMC) were cultured from the omentum of 8-week-old Wistar rats as previously described [7], and cultured in RPMI 1640 medium containing 10% fetal bovine serum. A retroviral vector pCMSCVpuro-16E6E7 was constructed by recombining the segment of a donor vector containing full-length HPV16E6 and E7 (a kind gift from Dr. Tohru Kiyono, National Cancer Center, Tokyo, Japan) into the destination vector by the Gateway System (Invitrogen Life Technologies, Carlsbad, CA) as described previously [8]. Briefly, RPMCs were infected at day 14 by the recombinant retrovirus expressing the 10A1 envelope with 4 µg/ml polybrene, and was drug selected using 1 µg/ml puromycin. NRK52E cells (Health Science Research Resources Bank, Osaka, Japan) were cultured in Dulbecco’s modified Eagle’s medium (GIBCO, Rockville, MD) containing 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B at 37°C in a humidified 5% CO₂ incubator.

Histology and immunohistochemical analysis
Frozen tissue specimens were fixed with 10% phosphate-buffered formalin at room temperature, and embedded in paraffin. Tissue sections with a thickness of 3-µm were stained with hematoxylin and eosin. For immunohistochemical analysis, the avidin-biotin complex method with peroxidase was used as described previously [9].

Antibodies
Anti-podoplanin polyclonal antibody (KS-17) was from Sigma (Saint Louis, MO). The concentrations used were 120 µg/ml for immunohistochemistry and 60 µg/ml for western blot analysis, respectively.
Western blot analysis

Lysates were prepared by homogenizing the samples in RIPA lysis buffer (20 mM Tris, 0.1% sodium dodecyl sulfate, 1% Triton X-100, 1% deoxycholate, pH 7.4) containing 0.2 mM sodium orthovanadate (Na$_3$VO$_4$), 50 mM/L sodium fluoride (NaF), 1 mM/L dithiothreitol and 5.7 µg/ml aprotinin. Western blot analysis was performed as described previously [10].

Results

To minimize the possibility of adipocyte contamination, we used only 6 to 8 week-old rats. At this age, the bulk of adipocytes surrounding the solid organs is low, and still each organ is large enough. We started by scraping at several different angles and depths, followed by histological confirmation of the scraped tissues. Scraping was done only once at each location to avoid taking parenchymal cells. We could obtain the best results when the scraped tissue came off rolling on the edge of the blade. Massive contamination of parenchymal cells was observed when we obtained apparent tissue fragments with scraping.

The amounts of protein obtained by using this method depended largely on the total surface area of organ scraped. Despite the fact that we did not scrape the whole surface of an organ, we still managed to obtain approximately 25 mg of proteins from a half of liver, 10 mg from one kidney and 8 mg from spleen.

In order to validate the collection of mesothelial cells from the surface of multiple organs using the novel technique, we performed histological, immunohistochemical and western blot analyses. Results from hematoxylin and eosin staining and immunohistochemistry showed that mesothelial cells were efficiently collected from the surface of various organs. Fig. 1Bb shows that mesothelial cells are absent at the scraped surfaces. As shown in Fig. 1Bd, a monolayer mesothelial cells lining the surface of organ was immunostained for podoplanin whereas scraped surfaces showed no positive immunostaining.

Western blot analysis was performed by the use of lysates of mesothelial cells scraped from the surface of spleen, liver and kidney as well as the remaining parenchymal tissues from the same organs. Lysates from both NRK52E (normal rat kidney cells) and RPMC E6E7 (normal rat mesothelial cells) cell lines were included as a negative control and a positive control, respectively. The analysis was done using anti-podoplanin antibody. Podoplanin is known to be expressed in mesothelial cells, lymphatic endothelium, glandular myoepithelial cells, ependymal cells and stromal reticular cells [11]. As shown in Fig. 1C, high levels of podoplanin was detected in the RPMC E6E7 cell line as a positive control, and in each of the collected mesothelial cell samples, whereas low level of podoplanin was present in the samples that consisted of the remaining parenchymal cells obtained from the different organs. Taking together, these results show that mesothelial cells were efficiently collected from fresh organs with this novel technique.

Discussion

The results demonstrated that the method we propose would be useful for the study of mesothelial cells in animals, especially for the pureness of the samples. Thus far, we and other investigators have tried various methods to obtain control samples of normal mesothelial cells in comparison to those from mesothelioma [12]. However, we have been satisfied with none of them. For example, we have tried to obtain animal mesothelial cells by scraping with tooth brush at room temperature after adding small amount of physiological saline solution. We could use this method, but the problem was the huge contamination of other cells and red blood cells. Iron release from hemoglobin might cause deleterious effects in some experiments. As an alternative strategy, we have used either omentum, mesentery or tunica vaginalis of the testis in rodents. The problem here is the contamination of adipocytes. By hardening the organs with freeze in liquid nitrogen, we succeeded in obtaining the almost pure population of mesothelial cells. It would be possible to apply this method to human samples as well.

Recent expression microarray techniques produce a large number of data in one experiment, and the right selection of genes is critical for the success of experiments and the advancement of science [13]. We hope that our novel but simple method would contribute to the understanding of human mesothelioma and ultimately its cure and prevention.

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Abbreviation

RPMC, rat peritoneal mesothelial cells.

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