Original Article *J. Clin. Biochem. Nutr.*, **46**, 265–268, May 2010

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

Li Jiang, Yoriko Yamashita and Shinya Toyokuni*

Department of Pathology and Biological Responses, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan

Received 12 January, 2010; Accepted 21 January, 2010; Published online 10 April, 2010

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.

*To whom correspondence should be addressed.
Tel: +81 52 744 2086 Fax: +81 52 744 2091
E-mail: toyokuni@med.nagoya-u.ac.jp

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

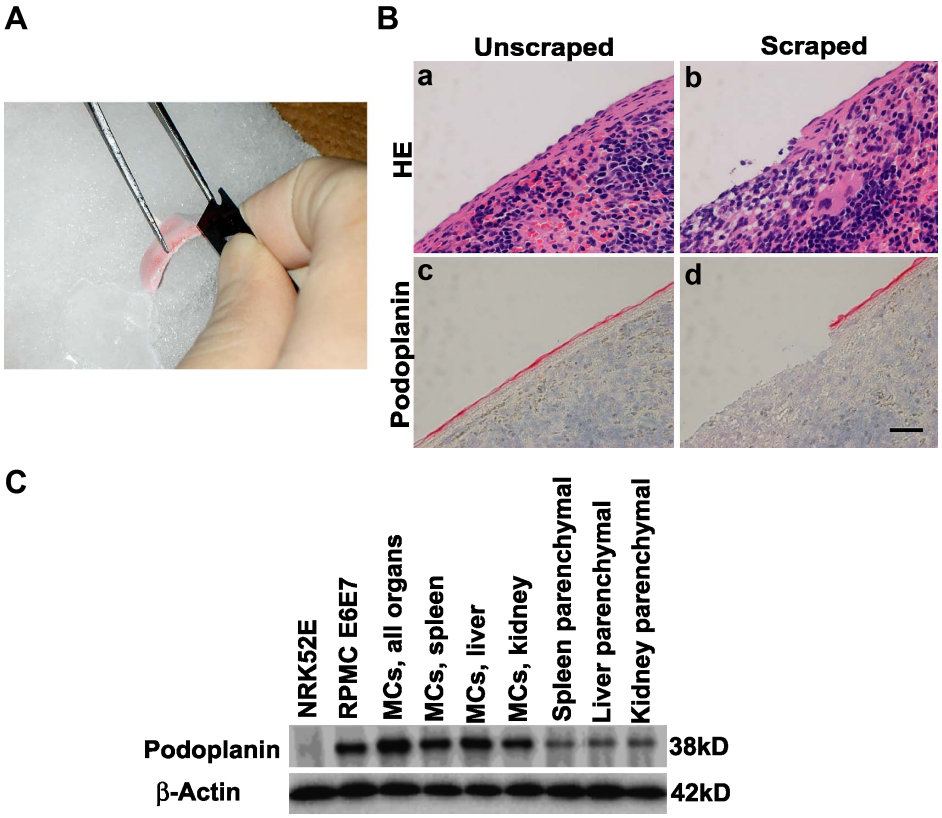

Fig. 1. A: The method to obtain pure mesothelial cells in animals. Rat spleen is snap-frozen in liquid nitrogen and is placed on dry ice. By the use of razor blades, we can obtain mesothelial cells by scraping. B: Histology and podoplanin immunohistochemistry (red) before and after scraping the surface of rat spleen (a–d). Samples once frozen were fixed in formalin at room temperature; a and c, and b and d are serial sections, respectively. With scraping, mesothelial cells are on the blades but not retained on the surface of spleen (b and d; bar = 50 μm). C: Western blot analysis for podoplanin. Collected mesothelial cells show high amounts of podoplanin. NRK52E, renal tubular cells (negative control); RPMCE6E7, immortalized rat peritoneal mesothelial cells (positive control). MC, mesothelila cells. Refer to text for details.

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% CO_2 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 mmol/L sodium orthovanadate (Na_3VO_4), 50 mmol/L sodium fluoride (NaF), 1 mmol/L dithiothreitol and 5.7 μg/ml aprotinine. 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.

Acknowlegments

This work was supported in part by a MEXT grant (Special Coordination Funds for Promoting Science and Technology), a Grant from Takeda Science Foundation, a Grant-in-Aid for Cancer Research from the Ministry of Health, Labour and Welfare of Japan, and a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Abbreviation

RPMC, rat peritoneal mesothelial cells.

References

[1] Jones, J.S.P., ed.: *Pathology of the mesothelium*, Springer-Verlag, Berlin Heiderberg, 1987.

[2] Churg, A., Cagle, P.T., and Roggli, V.L., eds.: *AFIP Atlas of*

Tumor Pathology, Series 4, Tumors of the serosal membranes, ARP Press, Silver Spring, Maryland, 2006.

[3] Robinson, B. and Lake, R.: Advances in malignant mesothelioma. *N. Engl. J. Med.*, **353**, 1591–1603, 2005.

[4] Toyokuni, S.: Role of iron in carcinogenesis: Cancer as a ferrotoxic disease. *Cancer Sci.*, **100**, 9–16, 2009.

[5] Toyokuni, S.: Mechanisms of asbestos-induced carcinogenesis. Nagoya *J. Med. Sci.*, **71**, 1–10, 2009.

[6] Cooke, M., Henderson, P., and Evans, M.: Sources of extracellular, oxidatively-modified DNA lesions: implications for their measurement in urine. *J. Clin. Biochem. Nutr.*, **45**, 255–270, 2009.

[7] Oravecz, T., Pall, M., Roderiquez, G., Gorrell, M., Ditto, M., Nguyen, N., Boykins, R., Unsworth, E., and Norcross, M.: Regulation of the receptor specificity and function of the chemokine RANTES (regulated on activation, normal T cell expressed and secreted) by dipeptidyl peptidase IV (CD26)-mediated cleavage. *J. Exp. Med.*, **186**, 1865–1872, 1997.

[8] Yamashita, Y., Tsurumi, T., Mori, N., and Kiyono, T.: Immortalization of Epstein-Barr virus-negative human B lymphocytes with minimal chromosomal instability. *Pathol. Int.*, **56**, 659–667, 2006.

[9] Toyokuni, S., Tanaka, T., Hattori, Y., Nishiyama, Y., Ochi, H., Hiai, H., Uchida, K., and Osawa, T.: Quantitative immunohistochemical determination of 8-hydroxy-2'-deoxyguanosine by a monoclonal antibody N45.1: its application to ferric nitrilotriacetate-induced renal carcinogenesis model. *Lab. Invest.*, **76**, 365–374, 1997.

[10] Toyokuni, S., Kawaguchi, W., Akatsuka, S., Hiroyasu, M., and Hiai, H.: Intermittent microwave irradiation facilitates antigen-antibody reaction in Western blot analysis. *Pathol. Int.*, **53**, 259–261, 2003.

[11] Schacht, V., Dadras, S., Johnson, L., Jackson, D., Hong, Y., and Detmar, M.: Up-regulation of the lymphatic marker podoplanin, a mucin-type transmembrane glycoprotein, in human squamous cell carcinomas and germ cell tumors. *Am. J. Pathol.*, **166**, 913–921, 2005.

[12] Hu, Q., Akatsuka, S., Yamashita, Y., Ohara, H., Nagai, H., Okazaki, Y., Takahashi, T., and Toyokuni, S.: Homozygous deletion of *CDKN2A/2B* is a hallmark of iron-induced high-grade rat mesothelioma. *Lab. Invest.*, **90**, 360–373, 2010.

[13] Takahashi, Y., Odbayar, T., and Ide, T.: A comparative analysis of genistein and daidzein in affecting lipid metabolism in rat liver. *J. Clin. Biochem. Nutr.*, **44**, 223–230, 2009.