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１．研究テーマ

LPS 刺激マウス胸膜中皮細胞の形態的変化及び細胞表面接着分子の発現が白血球の遊出に関与する機序

２．本年度の研究業績

(1) 学会・研究会等における発表 (有) ・ 無 (学会名・演題)

1、滕瑞峰、城倉浩平、崔麗、荻原直子、 佐々木克典：
LPS 刺激マウス胸膜中皮細胞及び遊出白血球に置ける細胞接着分子微細局在の三次元的観察
第 107 回日本解剖学会総会・全国学術集会、浜松、2002 年 3 月

2、Tetsuya Imamura, Ruifeng Teng, Yasumitsu Okouchi, Kohei Johkura, Naoko Ogiwara, Katsunori Sasaki: A new culture system imitating hepatic environment to induce hapatocytes from embryonic stem (ES) cell in vitro. 5th Annual Meeting of the Tissue Engineering Sociaty International, Kobe, December 8-10, 2002.

(2) 学会誌等に発表した論文 (有) ・ 無 (雑誌名・論文名)

1, Johkura K, Liang Y, Teng R, Ogiwara N, Sasaki K: Nephrogenesis accompanied by vascularisation in the mouse embryonic metanephros transplanted into the adult kidney for the creation of additional nephrons. Nephrology 7:92-100, 2002.

2, Teng R, Johkura K, Ogiwara N, Zhao X, Cui L, Iida I, Okouchi Y, Asanuma K, and Sasaki K: The Immunobiological Role of Pleural Mesothelial Cells in Neutrophil migration via Morphological Changes and Adhesion Molecules.
雑誌「Journal of Leukocyte Biology」に投稿中

—日中医学協会助成事業—

LPS 刺激マウス胸膜中皮細胞の形態的変化及び細胞表面接着分子の発現が白血球の遊出に関与する機序

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Abstract:

Pleural effusion is a common clinical problem arising as a result of many etiologies. Parapneumonic effusion caused by inflammatory pathogens and characterized by a massive protein exudation and leukocyte infiltration is associated with an increased morbidity and mortality. Examination and management of pleural fluid is an important clinical problem. However, our current understanding of the basic mechanisms by which fluid and leukocytes accumulate within the pleural space is very poor.

To clarify the mechanisms by which leukocytes penetrate across the pleural mesothelial layer, a mouse model with acute inflammatory pleural effusion triggered by intrapleural LPS injection was employed in this study. Normal and LPS-stimulated mouse parietal pleurae were investigated immunohistochemically, as well as with conventional transmission electron microscopy (TEM) and scanning electron microscopy (SEM). We found that normal parietal pleural mesothelial cells in the immediate vicinity of ribs were morphologically distinct from those in the other areas. Following LPS-stimulation, the mesothelial cells in these regions changed firstly from dome-shaped to spherical. The subsequent leukocyte penetration across mesothelial layer occurred primarily in these regions, too. Examinations of the expression of ICAM-1, VCAM-1, ICAM-2, PECAM-1, MAdCAM-1, ELAM-1, PNAd and fibronectin on parietal pleura were performed with immunological scanning electron microscopic (immuno-SEM) method. Only ICAM-1 was demonstrated to be constitutive on the parietal pleura and up-regulated with LPS stimulation. VCAM-1 was significantly induced. Their distribution on the mesothelial cell surface was restricted to the microvilli. The results from these morphological investigations prompted us to conduct a further investigation with blocking antibodies specific for ICAM-1, VCAM-1, LFA-1, Mac-1, VLA-4 and integrain β_2 chain. Blocking these cell surface adhesion molecules resulted in a marked inhibition (75.1%~97.9%) of neutrophil accumulation in the pleural space, which indicated that ICAM-1 and VCAM-1 were directly and essentially involved in leukocyte penetration through the mesothelial layer.

Based on this study, we conclude that the immediate vicinity of ribs in the parietal pleurae may be the source of leukocyte penetration into the pleural space when inflammation occurs there. The pleural mesothelial cells play an important role in this process because ICAM-1 and VCAM-1 are expressed on their microvilli.

Key Words:

pleural mesothelial cell; LPS; ICAM-1; VCAM-1; neutrophil.

Introduction

An array of studies concerning the influence of proinflammatory cytokines on human pleural

and peritoneal mesothelial cells cultured in vitro have yielded similar results, namely, that IL-1β, TNF-α and IF-γ can stimulate the mesothelial cells to express IL-8 as well as ENA-78, which is the C-X-C chemokine chemotactic for neutrophils [1,2,3,4,5,6,7,8], and MCP-1 as well as MIP-1α, which is the C-C chemokine chemotactic for monocytes [2, 6, 9]. The expression of ICAM-1 and VCAM-1, two members of the immunoglobulin superfamily on mesothelial cells, was found to be simultaneously upregulated in these studies [5, 6, 10, 11, 13]. Neutralizing chemokines of IL-8 and MCP-1 with specific antibodies resulted in a significant reduction in neutrophil and monocyte transmigration [5, 10]. When ICAM-1 expressed on the mesothelial cells was blocked, neutrophil transmigration across the mesothelial monolayer from the basolateral to the apical side was significantly inhibited too [5, 11,12]. The expression of VCAM-1 on the rat pleural mesothelial cells was also demonstrated recently [14].

These previous investigations seem to suggest that the pleural mesothelial cells may play an important role in leukocyte transmigration into the pleural space. They may facilitate leukocyte infiltration by inducing dysfunction of the mesothelial barrier, releasing chemokines chemotactic for leukocytes and expressing ICAM-1 and VCAM-1 on their surface. Although the expression of ICAM-1 and VCAM-1 on human and rat pleural mesothelial cells has been proved, how they are distributed on the cell surface remains unknown. Similarly, the questions of how they are involved in leukocyte penetration through the paracellular space and whether ICAM-1 and VCAM-1 function equally in this process have rarely been addressed.

Materials and Methods

For these experiments, 60 ICR male mice weighing 28-30g (SLC, Hamamatsu, Japan) were used.

Pleurisy was induced in 42 mice by intrapleural injection of lipopolysaccharide (LPS, Escherichia coli purchased from the List Biological Laboratories, INC, California, USA.). LPS powder was solubilized in water to a concentration of 250μg/ml. After successful anesthetization, a small incision (0.5-1.0cm) was made into the skin, and a volume of 0.16~0.18ml (1.5μg/gBW) of the LPS solution was injected with a micromanipulator into the murine left pleural cavity through the intercostal space. The incision was sutured after completion of the injection. For the eight negative controls, the same volume of normal saline (NS) instead of LPS was injected into the pleural space.

Blocking Treatment in vivo

A total of 14 mice were subjected to the blocking analysis. The antibodies used for neutralization, rat monoclonal antibodies (mAbs) against mouse ICAM-1, VCAM-1, LFA-1 and Mac-1 were purchased from Southern Biotechnology Associates, Inc (Birmingham, USA), and rat mAbs against mouse integrainchain, VLA-4 as well as normal rat IgG were purchased from BD Biosciences Pharmingen (San Diego, CA). All these purified antibodies were supplied in 1.0ml of 100mM borate buffered saline, PH 8.0, without the addition of any preservatives or amino-containing buffer salts. The 14 mice were divided into seven groups of two. At 6~8hr after intrapleural LPS-injection, each of the seven groups was anesthetized and administered one of the following seven antibodies: rat mAb against mouse ICAM-1 (250μg), VCAM-1 (250μg), ICAM-1 in combination with VCAM-1 (200μg/100μg), LFA-1 in combination with Mac-1 (150μg/150μg), integrain β_2 chain (100μg), integrain β_2 chain in combination with VLA-4 (100μg/150μg) and normal rat IgG (300μg). These antibodies were injected into the same sites where LPS had been introduced.

Experimental Design

Mice in group I (n=10) were normal control animals. Mice in group II (n=8) were treated with normal saline and sacrificed at 24hr. LPS-stimulated mice in group III (n=24) were sacrificed 1, 2, 8, 16, 24hr (eight mice in 24 hr and four mice in each of the other time points) after intrapleural LPS-injection. The remaining LPS-stimulated mice in group IV (n=18) were administered neutralizing antibodies or normal rat IgG and sacrificed at 24hr. All these animals were sacrificed under deep anesthesia induced with nembutal (1.0mg/20gBW). The pleural fluid samples were obtained following an intrapleural injection of 0.5ml normal saline before the pleural cavities were opened.

Results were analyzed with the aid of techniques of immunohistochemistry, transmission electron microscopy (TEM) and scanning electron microscopy (SEM).

Results

A small number of cells were detected in the normal pleural fluid where neutrophils were very infrequent. Both normal saline and LPS induced a marked influx of cells into the pleural cavity ($2.51 \times 10^4/\mu l$ and $9.56 \times 10^4/\mu l$). The average concentration of pleural fluid cells in the LPS-stimulated mice was about 2.8 times more than that in normal saline-stimulated mice, while neutrophils ($1.69 \times 10^3/\mu l$ and $8.96 \times 10^4/\mu l$) increased nearly 52 times, and accounted for approximately 93.7% of the total pleural fluid cells in LPS-stimulated mice.

One hour after LPS intrapleural injection, the mesothelial cells overlying the surface in the immediate vicinity of the ribs, which normally are dome-shaped, became spherical as if they had contracted. The intercellular junctions between these cells were disrupted and the cells separated completely. Microvilli on these cells aggregated from the peripheries to the convex central areas.

Significant leukocyte infiltration on parietal pleural surface was detected within 16 hr after LPS injection. The leukocyte recruitment on the parietal pleura surface was concentrated in the regions in immediate vicinity of the ribs. After 24 hr, some areas denuded of their mesothelial lining were observed.

Immunohistochemical Staining found that the LPS-stimulation induced significant up-regulation of ICAM-1 expression on the mesothelial and vascular endothelial cells of murine parietal pleurae in vivo. The dense distribution of capillaries in the connective tissues close to ribs was also confirmed.

In the LPS- stimulated parietal pleurae, on the other hand, abundant transmigrated neutrophils appeared both in the connective tissues and on the mesothelial surface close to the ribs. Staining for macrophages in the LPS-stimulated parietal pleurae showed only a scattering of positive cells among the numerous transmigrated neutrophils.

Immunogold-labeled ICAM-1, VCAM-1, ICAM-2, PECAM-1, MAdCAM-1, ELAM-I, PNAd and fibronectin on the normal and LPS-stimulated parietal pleurae and endocardia were examined with backscatter electron (BSE) imaging. Although the expressions of these adhesion molecules, except PNAd, ELAM-1 and fibronectin, were observed on both normal and LPS-stimulated endocardial cells, only ICAM-1 and VCAM-1 were detected on the parietal pleural mesothelial cells.

Administration of ample neutralizing antibodies for mouse ICAM-1, VCAM-1 and ICAM-1 combined with VCAM-1 in LPS-stimulated mice reduced neutrophil infiltration significantly compared to that in LPS controls. Combining of ICAM-1 and VCAM-1 had no additional effect. Sufficient

neutralization for LFA-1 combined with Mac-1 consistently led to a similar inhibition of neutrophil infiltration.

Discussion:

In our study, disruption of the intercellular junction in mesothelial cells was detected as early as 1hr after intropleural LPS-injection. The mesothelial cells in the immediate vicinity of the ribs where transmigration occurred later became spherical and had dense microvilli irradiating from their surface, giving them a hedgehog-like appearance. The tight membrane connections between these cells were completely disrupted. Intact basal lamina could be observed through the gaps between these separated mesothelial cells, but no transmigrated leukocytes on the mesothelial surface or underlying the mesothelial layer were observed at this time. In fact, transmigration of leukocytes across the mesothelial layer began between 8hr and 16hr after LPS-injection. This time lag between separation of mesothelial cells and emergence of leukocytes on the mesothelial surface precludes the possibility that leukocyte adherence to the mesothelium is a prerequisite for mesothelial cell disassociation.

In contrast to human peritoneal mesothelial cells which constitutively express ICAM-1, VCAM-1 and PECAM-1 on their surface [6, 15, 16], PECAM-1 was not detected on the murine pleural mesothelial cells with our immunoassay method. The expression of VCAM-1 was too feeble to be clearly distinguishable from that in negative control. We think this difference may be due to the difference in the species used in our study and in others.

Our study found that, where the adhesion molecules of ICAM-1 and VCAM-1 had clustered together, the LPS-stimulated murine pleural mesothelial cells became spherical with microvilli radiated from their surface. This suggests that it is a possible for activated pleural mesothelial cell to interact with the leukocytes traveling through the mesothelial layer with the aid of microvilli.

To confirm the participation of ICAM-1 and VCAM-1 in the process of leukocyte transmesothelial migration, LPS-stimulated mice were subjected to blocking. Significant inhibition of neutrophil transmigration into the pleural space was induced by sufficient neutralization for ICAM-1, VCAM-1, and ICAM-1 combined with VCAM-1, LFA-1 combined with Mac-1, β_2 chain combined with VLA-4 respectively. This finding leads us to conclude that both ICAM-1 and VCAM-1 play a pivotal role in neutrophil transmigration across the murine pleural mesothelial layer.

The source of leukocytes that have transmigrated into the pleural cavity has remained unclear. The observations obtained in our study indicate that the immediate vicinity of the ribs in the parietal pleurae are morphology specific regions. Mesothelial cells covering these regions in normal parietal pleurae are dome-shaped and PCNA positive. LPS stimulation resulted in morphological changes and the subsequent leukocyte transmigration across the mesothelial layer in most of these regions. Immunohistochemical staining for ICAM-1 showed strongly stained intercostals blood vessel networks that had located in the loose connective tissues of these regions. Massive leukocytes accumulated in these loose connective tissues and easily detectable leukocytes attached to the inner surface of the blood vessels in these regions were also observed. These visible signs strongly suggest that these regions in the immediate vicinity of the ribs in murine parietal pleurae were the source of leukocytes infiltrating into the pleural cavity.

In conclusion, the immediate vicinity of the ribs in normal murine parietal pleurae is thought to be the source of leukocyte filtration into the pleural space. Following LPS stimulation,

the mesothelial cells covering these regions first separated, which is a prerequisite for leukocyte transmigration across the pleural mesothelial layer. ICAM-1 and VCAM-1 were the only two adhesion molecules identified on murine pleural mesothelial cells. Blocking with neutralizing antibodies for ICAM-1, VCAM-1 and their counter-receptors on leukocytes produced significant inhibition of neutrophil infiltration into the pleural space. The mesothelial cells can thus be assumed to participate in the passage of neutrophils through the mesothelial layer by means of morphological changes and via the adhesion molecules localized on their microvilli.

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