Inflammation induced epithelial-mesenchymal/mesothelial cell-macrophage transition in mesenteric mesothelial cells

Doctoral theses

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Budapest
2015

Introduction

The mesothelium is an extensive monolayer, composed of mesothelial cells that line the serous cavities of the body and organs. The mesothelial cells secrete glycosaminoglycans and phosphatidylcholine to provide a non-adhesive, frictionless protective surface. They are able to transport fluids and particles trough the serosal membrane via stomata. Mesothelial cells can synthetize and secrete mediators in response to external signals, initiating and regulating an inflammatory response, recruiting cells into the serosal cavities and presenting antigen to T cells. They also play an important role in tissue repair through the release of cytokines and ECM molecules and their protease and fibrinolytic properties are of major importance in preventing fibrosis and adhesions (Mutsaers 2002).

The mesothelial cells are elongated, flat cells, they can be identified according their nuclei, and their cytoplasm is hardly seen and appears as wavy lines. Many flask- or omega-shaped, caveola-like plasma membrane invaginations are seen on both basal and apical sides. Only few intracellular organelles (mitochondria, endoplasmic reticulum and multivesicular bodies) presented in the cytoplasm. The mesenteric connective tissue between the two mesothelial layers contains only few cells (mainly fibrocytes, mast cells and granulocytes) collagen fibres and blood vessels (Katz et al 2011, 2012).

Intraperitoneal injection of Freund's adjuvant induces epithelial-mesenchymal transition (EMT) of mesothelial cells in rat's peritoneal cavity (Katz et al 2012). The EMT was described firstly by Elisabeth Hay in 1995 using a model of chick primitive streak formation (Hay 1995).

Steps of the epithelial-mesenchymal transition:

- loss of cell-cell and cell-matrix interactions,
- loss of polarity,
- basement membrane degradation,
- cytoskeletal rearrangement,
- migration.

Three subtypes of EMT can be distinguished with different functional consequences. Beside epithelial-mesenchymal transition during embryogenesis (type I) and tumorigenesis (type III), type II EMT is associated with wound healing, tissue regeneration and organ fibrosis (Kalluri and Weinberg 2009).

Mesothelial cell-macrophage transition?

Our laboratory's previous data showed the number of the peritoneal macrophages dramatically increased during Freund's adjuvant treatment (Kiss and Kittel 1995, Katz et al 2011). The exact origin of these macrophages is unanswered. Two possible sources are known: (a) the migration of monocytes from blood vessels into peritoneal cavity and (b) the activation of resident macrophages in omental "milky-spots". However, these two sources of newly appearing macrophages do not seem to explain the sharply increasing number of these cells from 10^5 /ml in the resting peritoneal cavity to 10^7 /ml in the inflammatory state. But the adjuvant treatment resulted remarkable morphological changes of mesothelial cells and their detachment from mesentery's surface strongly suggest that under special stimuli these cells can differentiate into macrophage or macrophage-like cells and they could be a third source of peritoneal macrophages.

GM-CSF and GM-CSF receptor

Granulocyte-macrophage-colony stimulating-factor (GM-CSF) stimulates the terminal differentiation of macrophages (Shiomi and Usui 2015). GM-CSF is a member of the hematopoietic cytokine family and promotes the survival and activation of granulocytes, macrophages and dendritic cell differentiation *in vivo*, but it also stimulates proliferation of several non-hematopoietic cell types (osteoblasts, smooth muscle, endothelial and epithelial cells) (Rasko and Grough 1994). The GM-CSF is produced by activated but not-resting T-lymphocytes (Gasson et al 1984), monocytes, fibroblasts, endothelial cells and stimulated keratinocytes (Metcalf et al 1986, Kupper et al 1988).

The cytokine was described to signal trough a heterodimeric receptor having a cytokine-specific α and common βc subunits (Martinez-Moczygemba and Huston 2003). The GM-CSF signalling is initiated by the cytoplasmic tyrosine kinase janus kinase 2 (JAK2), which acts on various downstream proteins. The principle signalling modules activated include the janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway, the mitogen-activated protein kinase (MAPK) pathway, and the phosphatidylinositol 3-kinase (PI3K) pathway and the canonical NF-κB transcription factors (van de Laar et al 2012).

Objectives

1. I followed the morphological changes of mesothelial cells during Freund's adjuvant induced inflammation.
2. To demonstrate the plasticity of mesothelial cells and the steps of cytoskeletal rearrangement of EMT during the inflammation I applied immunocytochemical analysis.
3. To induce the mesothelial cell-macrophage transition I worked out a primary mesenteric culture technique. These cultures were treated with GM-CSF. The GM-CSF induced changes were followed by light and electron microscopic techniques.
4. To study whether GM-CSF receptor is expressed on the mesothelial cells anti-GM-CSF receptor α was applied. To confirm the mesothelial cell-macrophage transition ED1 (pan-macrophage marker) labelling was used on primary mesenteric culture.
5. To detect if GM-CSF is present *in vivo* circumstances I applied Western Blot analysis with this cytokine in the peritoneal wash and the lysate of mesothelial cells during inflammation.
6. To demonstrate the presence of GM-CSF receptor in Freund's adjuvant treated samples *in vivo*, I applied immunocytochemistry with anti-GM-CSF receptor α. The mesothelial cell-macrophage transition was followed by studying the ED1 expression. To support my immunocytochemical data Western Blot assays were used to follow the expression of GM-CSF receptor and ED1 in mesothelial cells' lysate.
7. To study whether receptor-ligand internalisation is crucial for the mesothelial cell transition I followed the ED1 expression in the presence of Dynasore which inhibits the internalisation of membrane vesicles.

Methods

Material:

In vivo experiments: To induce peritonitis 1 ml complete Freund's adjuvant was injected into the peritoneal cavity of 70-90 days old male Sprague-Dawley rats (200-250g). After 1, 3, 5, 6, 7, 8, 9, 10, 11, 20 days the mesentery was isolated from control and treated animals.

For *in vitro* experiments a primary mesentery culture was developed: mesenteries were cut out from control animals, and maintained in Dulbecco's Modified Eagle Medium

Nutrient Mixture F-12 (DMEM/F12) in humid condition at 37℃ with 5% CO_2. The tissue cultures were treated either with 1 ng/ml GM-CSF 1, 2, 3, 6, 8 and 24h. To block endocytosis the primary mesenteric culture was pre-treated with 80μM Dynasore for 1h, then the media was supplemented with GM-CSF and cultures were incubated for 6 and 8h**.**

Applied methods:

- light- and electron microscopy
- immunocytochemistry
- Western Blot assay

Results

1) The intraperitoneal injection of Freund's adjuvant induces acute peritonitis in rat's peritoneal cavity. By the time of the adjuvant's treatment (at 3rd - 5th days of inflammation) the flat, simple squamous mesothelial cells became rounded, cuboidal shaped, they grow cytoplasmic processes towards to the mesenteric connective tissue, many of them have lost their connection with the neighbouring cells and detached from basement membrane. In their cytoplasm dramatically increase the number of cellular organelles (many mitochondria, well developed Golgi-apparatus, rough endoplasmic reticulum and multivesicular bodies), they lost their intercellular connections and polarity. The basal lamina disintegrated. At the 6th - 7th days of inflammation many autophagic vacuoles are in the cytoplasm of mesothelial cells, the cells are paralelly arranged on the mesenteric surface. Many free cells are presented close to the mesentery and the connective tissue becomes highly vascularised. At the 8th - 9th days the autophagic vacuoles are predominate in the mesothelial cell's cytoplasm, indicating that the reconstruction of the original morphology has already started. Between some cells the intercellular connections are detectable, the volume of mesothelial cells decreased. 11 days after the Freund's adjuvant treatment the inflammation is passed off. The mesothelial cells are flat, squamous cells, just few cytoplasmic organelles are presented, the basal lamina and their intercellular connections are integrated. The shape of mesothelial cells is similar to the non-treated cells.

2) To study the kinetic of EMT in mesothelial cells, I applied immunocytochemical analysis focusing on the reorganisation of the cytoskeleton. By the time of the Freund's adjuvant induced inflammation I followed the changes in the expression of cytokeratin (epithelial intermediate filament protein) and vimentin (a mesenchymal cell-specific

intermediate filament protein). I found that as the inflammation progressed the cytokeratin is gradually disappeared from the cytoplasm of the mesothelial cells and replaced by vimentin.

To check the plasticity of mesothelial cells, I detected Hsp47 and nestin antibodies. On the surface of the control mesentery, mesothelial cells were labelled with anti-nestin and anti-Hsp47. After the Freund's adjuvant treatment the expression of nestin and Hsp47 are considerably increased in all mesothelial cells.

3) To study the effect of GM-CSF on mesothelial cells *in vitro*, isolated mesenteries were held in DMEM/F12 culture medium for 1, 3, 6, 8, 12, 24, 48 hours. When treating the primary mesenteric culture with 1ng/ml GM-CSF (6 and 8 h) prominent changes could be detected: perinuclear areas of the mesothelial cells became more voluminous, and the amount of collagen fibres in the connective tissue has significantly increased. Some of the cellular junctions have already disappeared, and many cellular processes presented on the basal surface of the cells indicating that the mesothelial cells have started to lose their polarity.

4) In three days old non-treated mesentery cultures no ED1 positive mesothelial cells could be detected by immunohistochemistry. In contrast, 6 and 8 hours 1 ng/ml GM-CSF treatment induced a well observable expression of ED1 (a characteristic macrophage marker) in these cells. To answer the question whether internalization has an essential role in the GM-CSF signalling driving the cell to express ED1, we used Dynasore. Dynasore is known to block pinching off clathrin-coated vesicles, caveolae and some lipid rafts from the plasma membrane (Fletcher et al 2012). Presence of Dynasore (before and during the whole experimental period) was inhibited the ED1 expression in mesenteric mesothelial cells.

To mediate the biological effect of GM-CSF, receptor is supposed to be present on the target's cell membrane. My immunocytochemical results show that GM-CSF receptor is really presented in the 3 day cultured mesenteric mesothelial cells (even if the GM-CSF was not present). In mesenteries maintained in culture for three days and treated consecutively with GM-CSF for 8 hours, I found a significantly increased receptor expression. A prominent labelling could be detected in the cytoplasm.

5) Studying the level of GM-CSF in the *peritoneal wash* by Western Blot technique I found maximum level of this cytokine at the peak time of inflammation (5th and 8th days). However, low levels of this hematopoietic factor were present even in healthy and

regenerated conditions, suggesting that there is a steady state level of GM-CSF in the peritoneal cavity.

To detect whether mesothelial cells *in vivo* can produce and secrete GM-CSF into the peritoneal cavity, I also used Western Blot assay. In the *mesothelial cell extract* I found that the control cells did not express GM-CSF, but the level of this cytokine considerably increased by the 3rd and the 5th days of inflammation. As the regeneration of mesentery has started (8th day), the amount of the GM-CSF was decreased to reach a level similar to that of the control after a few days.

6) The GM-CSF receptor was localized with immunohistochemistry on frozen semithin sections. Although it is difficult to determine the precise localization of the receptor in mesothelial cells of non-treated animals due to their highly flattened shape, my immunocytochemical confocal images suggest that the receptor is present mainly on the plasma membrane of the cells. By the 3rd day of inflammation when the mesothelial cells become more voluminous, many small immunopositive punctate structures appeared in the cytoplasm. At the 5th day of inflammation the number of GM-CSF receptor positive structures was lower in the cytoplasm, but they were increased in size. Time sequence of the GM-CSF-receptor expression was followed on Western Blots of mesothelial lysates, which clearly indicated a relatively low level of GM-CSF receptor expression in control samples and a sharp rise by 5th day (maximum of inflammation) with a subsequent decrease during regeneration.

ED1, a macrophage marker and indicator of mesothelial cell transformation was followed on Western Blots of mesothelial lysates and with immunocytochemistry. While immunoblots from control animals gave no signal for ED1, the macrophage marker was expressed on 3rd day of inflammation and reached a maximal level on 5th day, coinciding with the maximum of the inflammation reaction. Immunocytochemical localization of ED1 on transformed mesothelial cells gave a strong signal primarily along the plasma membrane.

Conclusions

The peritoneal injection of Freund's adjuvant results in remarkable changes in the morphology of the mesothelial cell lining the surface of rat mesentery. The inflammatory stimulus induced an extensive cellular proliferation, migration, structural reorganisation and loss of cell continuity. Since Freund's adjuvant causes massive inflammation and inflammatory cytokines initiate mesothelial transdifferentiation, my morphological data suggest that the adjuvant treatment induces epithelial-mesenchymal transition (EMT). My detailed immunocytochemical experiments (expressional changes of cytokeratin, vimentin, Hsp47 and nestin) verified this transition.

Freund's adjuvant induced acute peritonitis in rat results a prominent, significant increase in the number of peritoneal macrophages. It is known that during inflammation monocytes, migrating out from the blood vessels, and macrophages, resting in the „milky spots" of the peritoneum, become activated and play important role in the local defense. But these two sources of newly appearing macrophages do not seem to explain the huge number of these cells. We think a further source of this increased phagocytic cell population could be the mesenteric mesothelial cells by epithelial-mesenchymal transition. During this process mesothelial cells can be detached from the underlying basement membrane, and assume a macrophage character both morphologically and by expressing macrophage markers. To induce mesothelial cell-macrophage transition GM-CSF was used on primary mesenteric cultures *in vitro*. As a result of GM-CSF treatment the mesothelial cells lost contact with each other and with the underlying basal lamina, basal cell processes appeared and cells became more voluminous, and started to express macrophage marker (ED1). This indicates that GM-CSF was sufficient to induce mesothelial cells-macrophage transition. My immunocytochemical data show mesothelial cells express GM-CSF receptor both *in vivo* and *in vitro* circumstances. The Western Blot results already show that mesothelial cells can produce and secret GM-CSF cytokine into the peritoneal cavity, indicating that they can regulate the mesothelial cell transition in an autocrine way.

My *in vitro* and *in vivo* experiments provide persuasive evidence that during inflammation mesenteric mesothelial cells undergo epithelial-mesenchymal transition. Parallel to this process they can differentiate into macrophage or macrophage-like cells. All the factors (receptor and cytokine) necessary for this transition are present and produced on/by mesothelial cells.

Own publications

Related articles:

Balogh P, Katz S, Kiss AL. (2012) The role of endocytic pathways in TGF-β signalling. Pathol Oncol Res, 19 (2): 141-148.

Balogh P, Szabó A, Katz S, Likó I, Patócs A, Kiss AL. (2013) Estrogen receptor alpha is expressed in mesenteric mesothelial cells and is internalized in caveolae upon Freund's adjuvant treatment. PLoS One, 8 (11): e79508. doi: 10.1371/journal.pone.0079508.

Katz S, Balogh P, Nagy N, Kiss AL. (2012) Epithelial-to-mesenchymal transition induced by Freund's adjuvant treatment in rat mesothelial cells: a morphological and immunocytochemical study. Pathol Oncol Res, 18 (3): 641-649.

Katz S, Balogh P, Kiss AL. (2011) Mesothelial cells can detach from the mesentery and differentiate into macrophage-like cells. APMIS, 119 (11): 782-793.

Unrelated articles:

None.