The role of the internalization of GM-CSF receptor β and the autophagy in the regulation of EMT and MET

Doctoral thesis

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1. Introduction

The epithelial-mesenchymal transition (EMT) is a biological process in which polarized epithelial cells differentiate into cells with mesenchymal phenotypes through morphological and molecular changes: they lose their polarity, their connection with neighbouring cells and the basement membrane; their cytoskeleton is reorganized; their gene expression pattern changes and they will be able to migrate. The mesenchymal-epithelial transition (MET) is the reverse of EMT, as a result, mesenchymal cells regain their epithelial properties.

In our previous work we confirmed, that during the Freund's adjuvant induced inflammation rat's mesenteric mesothelial cells losing their epithelial feature differentiate into mesenchymal, macrophage-like cells (EMT II), then the regeneration (MET) starts after the peak of inflammation, as a result, cells regain their original simple squamous phenotype by the 11^{th} day after injection.

Many factors can induce EMT and MET. One such factor is the immunomodulator, granulocyte-macrophage colony-stimulating factor (GM-CSF) playing an important role in haematopoiesis as well. GM-CSF plays a crucial role in the maturation of dendritic cells; in differentiation, survival and activation of granulocytes and macrophages; in addition, it also has many non-haematopoietic functions that contributes to inflammatory responses.

Several cell types can produce GM-CSF. In our previous studies we confirmed, that during the Freund's adjuvant induced

inflammation mesothelial cell also produce GM-CSF. GM-CSF binding to its heteromeric receptor complex can exert its effect. The receptor complex is composed of a ligand-specific and ligand-binding α subunit (GM-CSFR α), as well as a β subunit (GM-CSFR β) which is responsible for signal transduction. Both subunits of the receptor can be expressed by many cells, like mesothelial cells.

GM-CSF-induced signalling pathways are well known. However limited data are available whether the internalization of receptor is necessary to initiate these signalling processes. Although there are literary data describing the internalization of GM-CSF receptor, these suggest, that the initial activation events of signal transduction occur without the internalization of receptor. In contrast, our previous results clearly showed, that if the dynamin-dependent internalization of receptor is inhibited by dynasore treatment, GMT-CSF treatment does not lead to EMT. Therefore, during my PhD work, I examined the internalization of GM-CSFR β which is responsible for initiating GM-CSF signalling and its cytoplasmic pathway following the internalization.

The other major topic of my work was the study of molecular processes involved in the regeneration of mesothelial cells. Our preliminary morphological and morphometric results showed, that during the regeneration, following the inflammation, mesothelial cells remove the unnecessary cellular organelles, cytoplasmic components, proteins by progressive autophagy. Autophagy is a physiological process by which the cells break down their own proteins, substances, and organelles by lysosomal degradation.

Thus, autophagy plays key role in cell survival, tissue regeneration, maintaining cellular homeostasis, inflammatory processes, immunity and cellular stress response as well. The molecular machinery of autophagy (from initiation to degradation) suggests, that the regulation of the process is extremely complex. One of the key autophagy-regulating signalling processes is the PI3K-Akt-mTOR pathway, in which the activated mTOR complex inhibits autophagy. Therefore, I studied how the expression and activation of these factors are involved in the regulation of autophagy (Akt/p-Akt, mTOR/p-mTOR, EEA1, Rab7, Beclin-1 (Atg6) and LC3B (Atg8)) during inflammation and recovery. The question was whether regeneration occurred if the autophagy was inhibited, and the main MET-inducers, the BMP proteins are involved in regeneration of mesothelial cells as well.

2. Objectives

1.) During inflammation mesothelial cells produce GM-CSF cytokine and express its receptor. Our *in vitro* experiments confirmed that GM-CSF and/or TGF-β treatment induces similar phenotypic transition as Freund's adjuvant. Our preliminary experiments suggested that the internalization of receptor-ligand complex is essential for the signalling process of GM-CSF. Therefore, I tried to answer the following questions:

- Do mesothelial cells express the GM-CSF receptor β, which is responsible for initiating signalling pathways?
- How GM-CSFR β is internalized, what is the further pathway of the internalized receptor (early, late and recycling endosomes) during the signal transduction?
- Since STAT5 transcription factor plays important role in GM-CSF signalling, I followed the expression and the cellular distribution of the active (phosphorylated) form of STAT5 in mesothelial cells.
- Is there any negative regulator in mesothelial cells that stops the process of EMT by blocking GM-CSF signalling pathways?

2.) I also studied the role of autophagy in the regeneration of mesothelial cells. I wanted to answer the following questions:

- How do various markers, indirectly (Akt/p-Akt, mTOR/p-mTOR, EEA1, Rab7) and directly (Beclin-1, LC3B) involved

in autophagy are expressed at different times of inflammation and regeneration?

- Are the MET-inducing BMP proteins and their receptors expressed in mesothelial cells?
- If the autophagy is inhibited by bafilomycin A1 treatment can the regeneration occur in mesothelial cells?

3. Methods

3.1. Animal models

In *in vivo* experiments, 60-70 days (200-300 g) male Sprague-Dawley rats (Charles River Research Models and Services, Sulzfeld, Baden-Wurttemberg, Germany) were used.

3.2. Materials

To induce acute peritonitis 1 ml complete Freund's adjuvant (Sigma-Aldrich®, Saint Louis, Missouri, USA), to inhibit autophagy bafilomycin A1 (Santa Cruz Biotechnology, Inc., Dallas, Texas, USA) was injected into the peritoneal cavity of the animals.

3.3. *In vivo* experiments

Mesentery was isolated at various time after Freund adjuvant treatment (3, 5, 8, 11 days), and were used for further experiments. Mesentery of untreated animals was used as control.

3.4. *In vitro* experiments

For all *in vitro* experiments mesentery was cut out from control rats, maintained in DMEM/F12 medium, and treated with 1 ng/ml GM-CSF (Sigma-Aldrich®, Saint Louis, Missouri, USA). In some cases, to inhibit dynamin-dependent endocytosis, primary mesenteric cultures were pre-treated with 80 μM dynasore (Sigma-Aldrich®, Saint Louis, Missouri, USA).

3.5. Morphological, light and electron microscopic examinations

3.5.1. Fixation and preparation of the mesentery

Mesentery was fixed with a 1:1 mixture of 2% glutaraldehyde and 2% osmium tetroxide, it was washed with cacodylate buffer, then the adipose tissue around the mesentery was removed. After washing, the samples were dehydrated with rising alcohol, and embedded in araldite. For light microscopical experiments, semithin sections were cut from the embedded samples by Reichert ultra-microtome, stained with toluidine blue, and examined by conventional light microscope (Carl Zeiss), photographed with Zeiss Axiocam HRc camera, and analysed with Axiovision. For electron microscopical experiments ultrathin sections were prepared, contrast-stained with uranyl acetate and lead (II) nitrate, the samples were examined by Hitachi H-7600 transmission electron microscope.

3.5.2. Immuno-electron microscopy

For immunocytochemical experiments, our samples were fixed in 1% PFA and after washing embedded in gelatine. Cryoprotection was done by incubation in 2,3 M sucrose, then the blocks were mounted onto aluminium pins and stored frozen in liquid nitrogen. Frozen ultrathin (70 nm) sections were cut by Leica Ultracut S ultramicrotome. The sections were washed with glycine-PBS, then blocked with 1% BSA and incubated for 1 hour with anti-GM-CSFR β antibody. For double labelling anti-EEA1, anti-Rab7 antibodies were also used. To visualize GM-CSFR β 10 nm colloidal gold conjugated protein A (PAG10) was used, while EEA1 and Rab7

were detected by 15 nm colloidal gold conjugated protein A (PAG15). The sections were contrast-stained with 2% uranyl acetate and incubated in a mixture of 1,8% methylcellulose and 0,4% uranyl acetate. The samples were examined in Hitachi H-7600 transmission electron microscope.

3.6. Confocal microscopy

3.6.1. Sample preparation for confocal immunocytochemical studies

For immunolabelling on frozen sections, we applied a modified Tokuyashu technique. The PFA-fixed samples were washed, and embedded in 10% gelatine (30 min, 37°C), then small blocks were formed. For cryoprotection, blocks were incubated in 2,3 M sucrose (overnight, 4°C), after mounted onto aluminium pins and frozen in liquid nitrogen. The 0,6 μm thick frozen sections were prepared by Leica Ultracut S ultramicrotome

3.6.2. The process and evaluation of fluorescent immunolabelling

Our semi-thin sections were washed with glycine-PBS and then blocked with 1% BSA-PB, incubated with the primary antibodies overnight (4°C). To visualize the primary antibodies, species-specific Alexa Fluor conjugated antibodies (Alexa Fluor 488, 1 hour, dark) were used. For double labelling experiments the sections were washed again with glycine-PBS, blocked with 1% BSA-PBS, and the above-mentioned steps were repeated with the appropriate secondary antibodies and their specific Alexa dyes (Alexa Fluor 555). Nuclei were stained by Vectashield DAPI. The sections were examined by confocal microscopy Zeiss LSM-780

(Carl Zeiss Technika Kft., Budaörs, Hungary) and analysed by ZEN program. Then the images were processed with Adobe Photoshop 7.1 software.

3.7. Statistical analysis

Our immunocytochemical investigations were supported by statistical measurements. To determine the colocalization of the different markers, 12 mesothelial cells were analysed in every test group using the ZEN program. The obtained correlation coefficients were analysed with descriptive statistics. All results were reported as mean ± SD (SD).

3.8. Biochemical experiments

3.8.1. Isolation of mesothelial cell lysate, cytoplasm and nuclear fractions

Control and Freund's adjuvant injected peritoneal cavities were washed with PBS, then isolated mesentery was incubated in 0,2% collagenase (Sigma-Aldrich®, Saint Louis, Missouri, USA) at 37^{O}C. After collagenase digestion, the samples were washed interposing centrifugations (1000 rpm, 3 x 10 min, 4°C), and stored at -80° C until further processing. The isolated mesothelial cell lysates were dissolved in lysis buffer, then centrifuged (12000 rpm, 20 min, 4°C). The protein content of the supernatant (mesothelial cell lysate) was determined by the BCA method and the samples were diluted to 2 mg/ml. To isolate nuclear and cytoplasmic fractions the mesothelial cell lysate was centrifuged at 1000 rpm and

then 2000 rpm, the precipitate was resuspended in 500 µl hypotonic buffer, and 25 µl detergent (10% Nonidet P-40) was added to the samples. After centrifugation, the supernatant (cytoplasmic fraction) was collected and stored at -80 °C. The pellet (later nuclear fraction) was resuspended in 50 µl complete Cell Extraction Buffer (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA), and incubated for 30 minutes on ice. After centrifugation (14000 g), the supernatant (nuclear fraction) was stored at -80 °C. The protein content of the two fractions were determined by the BCA method, and the samples were diluted to 2 mg/ml.

3.8.2. Western blot analysis

Tris-SDS buffer was added to the samples (in 1:1 ratio), and these were boiled for 4 minutes. Subsequently, the proteins were separated by electrophoresis (10% polyacrylamide gel, 200 V, 30-40 min). To transfer the proteins to the nitrocellulose membrane the gel and membrane were blotted at 100 V for 1 hour under continuous cooling in blot buffer. The membranes were washed in 0,0005% Tween-PBS buffer, then the membranes were stained with Ponceau S (Sigma-Aldrich®, Saint Louis, Missouri, USA) for instant visualization of the protein fractions, and blocked with 5% non-fat dry milk, washed, and incubated with the primary antibodies (overnight, 4°C). β-tubulin, β-actin and lamin A were used as an internal loading control. The membranes were incubated with specific peroxidase-conjugated secondary antibodies (Amersham, GE Healthcare Biosciences, Pittsburgh, USA) for 2 hours to visualize the primary antibodies. For detecting the labelled proteins,

the membranes were treated with a chemiluminescent solution (Luminata Forte Western HRP Substrate, Millipore, USA), and the immune response was detected using X-ray film or LI-COR C-Digit Blot scanner. The relative densities for the evaluation of the obtained Western blot data were determined from the results of 3 independent measurements using the ImageJ program.

4. Results

4.1. The internalization of GM-CSFR β in mesothelial cells: immunocytochemical and quantitative studies

4.1.1. The expression and internalization of GM-CSFR β: examination of caveolin-mediated endocytosis in vivo and in vitro

We have demonstrated that GM-CSFR β is expressed during inflammation. By the time of inflammation its expression is significantly increasing, but gradually decreasing after the peak time of inflammation.

Our morphological and statistical experiments prove that the internalization of GM-CSFR β is occurred by caveolae.

4.1.2. The pathway of the internalized GM-CSFR β towards early endosomes

Our double-labelled immunocytochemical experiments (both on frozen semi- and ultrathin sections) as well as our statistical analyses showed that as the inflammation proceeded the GM-CSFR β could be detected in EEA1-positive early endosomes. On the 3rd day of inflammation GM-CSFR β could be found in the membranes of early endosomes, supporting the idea that the internalized receptor is transported from the caveolae into early endosomes.

4.1.3. Further fate of GM-CSFR β: recycling or degradation?

We also found that till the inflammation persists, the receptor was present in Rab11a-positive recycling endosomes in mesothelial cells, indicating that a significant amount of the receptor recycled back to the plasma membrane, and could take part in

another endocytic cycle, maintaining the inflammation induced EMT. When the regeneration started, increasing amount of receptor could be detected in Rab7-positive late endosomes, indicating that lysosomal degradation of GM-CSFR β started.

4.2. Signal transduction during EMT and MET: activation of STAT5

Our immunocytochemical and Western blot results showed that in control mesothelial cells STAT5 is already phosphorylated on tyrosine residue (p-STAT5). The expression of this active form is significantly increased by the 3rd day of inflammation and large amounts of p-STAT5 could be detected both in the cytoplasmic and nuclear fractions of the cells. On the 5th day of inflammation the expression level of p-STAT5 slightly, then drastically decreased till the end of regeneration (day 11).

When dynamin-dependent endocytosis of GM-CSFR β was inhibited by dynasore treatment, no active, p-STAT5 could be detected in mesothelial cells. These results provide persuading evidence that the internalization of the receptor β subunit is essential for GM-CSF signalling.

4.3. The expression of SOCS1

Our immunocytochemical results showed that SOCS1 was expressed both in control and treated mesothelial cells and was found in diffuse distribution in the cytoplasm. On the 3rd day of inflammation, however, its expression decreased, indicating that

SOCS1 had no inhibitory effect on GM-CSF signalling, and the JAK2-mediated STAT5 phosphorylation could occur.

4.4. Autophagy

4.4.1. Activation of Akt and mTOR

We found that on the 3rd day of inflammation, the level of phosphorylated Akt reaches the maximum resulting in a significant increase in the expression of p-mTOR as well, indicating that during the time of progressive inflammation, the highly expressed active p-Akt and p-mTOR inhibit autophagy in mesothelial cells. After the peak of inflammation, however, expression of p-Akt, and p-mTOR is dramatically decreased, which allowed the autophagy to speed up.

4.4.2. Expression of Beclin-1 and LC3B

In the early stage of inflammation (day 3), the expression of Beclin-1 is significantly increased and then gradually decreased from day 8, indicating that one of the necessary factors for the induction of autophagy is present in our system.

One of the important membrane markers of autophagic organelles is LC3B. The results of our biochemical analyses clearly showed that as the regeneration progressed, the amount of membrane bound LC3B gradually decreased, indicating that the membrane bound LC3B was also degraded by progressive autophagy.

4.5. The expression of BMP proteins and their receptor in mesothelial cell

Our biochemical results showed that MET-inducing BMP4 is already expressed on the 3rd day of inflammation in mesothelial cells. It reached its maximum on the 5th day, although as the recovery started, its level slightly decreased, but mesothelial cells still expressed it on the 11th day. Control mesothelial cells express a small amount of BMP ligand-specific receptor, BMPR2. After Freund's adjuvant treatment, however, the expression of the receptor gradually increased until the end of regeneration.

4.6. The effect of bafilomycin A1 on the regeneration of mesothelial cells

Our light and electron microscopical results showed that bafilomycin A1 treatment itself has no effect on the morphology of mesothelial cells. On the 3rd and 5th days of inflammation mesothelial cells, taken from bafilomycin A1 treated animals showed the characteristic morphological signs of apoptosis. Freund's adjuvant and single and/or combined bafilomycin A1 (8 days) treated mesothelial cells showed no regeneration, instead the inhibition of autophagy resulted in apoptosis and cell death in mesothelial cells. These results clearly prove that autophagy is the key process in regeneration of mesenteric mesothelial cells.

5. Conclusions

- Our experiments proved that rat mesenteric mesothelial cells express the β subunit of GM-CSFR, which is responsible for signal transduction in GM-CSF signalling. Its expression is significantly increased during inflammation.
- The internalization of GM-CSFR β is indispensable for initiating signalling processes. GM-CSFR β is internalized by caveolin-mediated endocytosis in mesothelial cells and follows the classical early-late endosome, lysosome pathway.
- The phosphorylation of JAK2-mediated STAT5 on tyrosine residue most probably occurs in early endosomes. Blocking of dynamin-dependent endocytosis of GM-CSFR β inhibits the phosphorylation of STAT5, certifying that without receptor internalization there is no GM-CSF signalling.
- Until the peak time of inflammation the GM-CSFR β recycling is significant, maintaining the inflammation-induced EMT.
- As the regeneration starts GM-CSFR could be detected in late endosomes, indicating that the receptor degradation is already started. If the signal transmitting receptor β is degraded, the regeneration (MET) of mesothelial cells could start.
- We confirmed that SOCS1 regulatory protein is expressed in mesothelial cells, providing the negative feedback and stops GM-CSF signalling.
- We confirmed that for the regeneration of mesothelial cells autophagy is essential. The expression of Beclin-1 and LC3B -

which are directly involved in the process of autophagy - changes according to the dynamics of autophagy.

- In the early stages of inflammation, the autophagic processes are arrested by the increased expression of the negative regulators (p-Akt, p-mTOR) of autophagy. However, after the peak of inflammation, p-Akt and p-mTOR are inactivated, autophagy can speed up, making possible the regeneration of mesothelial cells.
- The regeneration of mesothelial cells (MET) is promoted by BMP factors. At the early time of inflammation mesothelial cells express BMP4 that most probably compensates EMT in the presence of BMPR2 and induces MET by auto-paracrine regulation. BMP7 is not expressed by mesothelial cells but is present in the abdominal cavity and its amount gradually increases by the time.
- Mesothelial cells of the animals treated with bafilomycin A1 at different days of inflammation, are not able to recover, they die by apoptosis, confirming that autophagy is indispensable in the post-inflammatory regeneration of mesothelial cells (MET).

6. Bibliography

6.1. Publications related to the dissertation:

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6.2. Other publications:

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