The possible role of estradiol and estrogen receptor alpha in TGF-β induced type II epithelial-mesenchymal transition and the following regeneration in mesenteric mesothelial cells

PhD thesis

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1. Introduction

Epithelial-mesenchymal transition (EMT) is a biological process that allows a polarized epithelial cell to undergo several biochemical and morphological changes to assume a mesenchymal phenotype (Kalluri&Weinberg 2009). The process of EMT was first described by Elisabeth Hay who also depicted the basic differences of the cellular actions observed during embryogenesis and tumorigenesis (Hay 2005). Since then three subtypes of EMTs have been distinguished with different functional consequences. Besides epithelial-mesenchymal transition during embryogenesis (type I) and tumorigenesis (type III), type II EMT is associated with wound healing, tissue regeneration and organ fibrosis (Sodek et al 2012, Yanez Mó et al 2003). It has been demonstrated that upon inflammation many cells (monocytes/macropaghes, fibroblasts) can trigger type II EMT through secretion of growth factors such as transforming growth factor-beta (TGF-β) or epidermal growth factor (EGF). TGF-β was first described to induce EMT via the canonical Smad 2/3-dependent pathway and it also became evident that the cellular actions of the cytokine can be modulated by other Smad-independent signaling pathways like MAP kinase pathways (Zavadil&Böttinger 2005, Derynck&Zhang 2003, Massagué 1998). The universal role of TGF-β in the different types of EMTs is unambigous as well as the biochemistry of the signaling is well characterized. It is less clear, however in which cellular/cytoplasmic compartments the molecules along the downstream pathway are accommodated and how their localization changes with the dynamics of the signaling. Another question of great interest is whether different compartments can be involved in regulating the pathway and if so, whether they can promote or suppress the signaling events?

There are two main endocytic pathways through which the TGF-β ligand-receptor complex can be internalized. One of them is the well-characterized clathrin-mediated endocytosis and a less characterized pathway is the lipid/caveola-mediated endocytosis. Both types of pathways are used for the internalization of TβRs (Di Guglielmo et al 2003).

In the classical clathrin-dependent pathway, after internalization the receptor-ligand complex is targeted to EEA1 positive early endosomal compartments. These endosomes maintain special environment where the phosphorylation of the signaling molecules

(Smad 2/3) is facilitated with the help of proteins like SARA. The central compartment in the enhancement of signaling inevitably are the early endosomes. It has also been proved that TβRs are accommodated in caveolin-1 containing lipid rafts of the plasma membrane and use the caveola mediated internalization route as well. Caveolae are small omega or flask-shape plasma membrane invaginations that play important role in many cellular functions including endocytosis, signal transduction, cellular growth control and apoptotic cell death (Couet et al 1997, Lisanti et al 1994).

The lysosomal degradation of internalized cargos includes multivesicular body (MVB) formation and the early endosomes are presumably the clue compartments of this process as well. Internalized cargo proteins are targeted first to early endosomes (Hayer et al 2012). MVBs are formed when limiting membrane of endosomes invaginates and buds into the lumen of the organelles. (Gruenberg&Maxfield 1995, Felder et al 1990). A subset of membrane proteins within the limiting membrane of the endosomes are sorted into these invaginating vesicles and this sorting requires the inclusion of a 350 kDa complex, called ESCRT (endosomal sorting complexes required for transport). The ESCRT complex containing endosomal membrane domains recognize and bind ubiquitinated cargos and initiate the transport of the proteins to late endosomes/multivesicular bodies. MVB sorting into intraluminal vesicles (ILV) and the subsequent lysosomal degradation of cell surface receptors is therefore a critical mechanism for regulating the signaling events (Katzmann et al 2001). Furthermore, the asymmetric compositon of the limiting membrane of these endosomal compartments presumably provide a platform for generating unique signals as well (Hanson&Cashikar 2012). Hence, early endosomes play a central role not only promoting the TGF-β pathway, but it is likely to be important intermediate cytosolic compartments that help to attenuate the signaling as well.

Recently, estrogen receptor alpha (ER-α) has been suggested as another player in the molecular mechanism of EMT (Guttila et al 2012, Ye et al 2010, Planas-Silva&Waltz 2007). For long, estrogen receptors (ER-α, ER-β) have been considered exclusively as transcription factors acting inside the nucleus (Beato et al 1995). However, the discovery of its membrane-associated form and the ER–mediated transcription in the absence of its ligand generally changed this concept (Driggers&Segards 2002, Levin 2002). The theory of a hormone-independent ER-α

activation that can serve as a mechanism to amplify growth factor pathways (Hall et al 2001) has also been accepted by now. Besides the nuclear and cytoplasmic pool of ER-α, it has been proved that a small percentage of the receptor (5-10%) resides in the cell membrane and can elicit both genomic and non-genomic responses by activating multiple protein kinase cascades that include MAPK, protein kinase C, Src kinase and PI3K (Levin 2009, Song&Santen 2006, Song et al 2005, Simoncini et al 2004, Razandi et al 1999, Migliaccio et al 1996, Pietras&Szego 1977).

The natural ligand of ER-α, estrogen is considered an important morphogen. Meanwhile the concept about ER receptors has largely changed, the renewal of the theory about their ligand effects was essential as well. Besides their gonadal synthesis some articles reported extragonadal estradiol (E2) production ex. in adipocytes, osteoblasts (Bruch et al 1992), chondrocytes, vascular endothelial cells (Bayard et al 1995), aortic smooth muscle cells (Murakami et al 1998) and brain tissue (Labrie et al 1997). Thus E2 is no longer solely an endocrine factor, but produced in several extragonadal sites, it has the potential to exert its biological effects locally acting as a paracrine or intracrine factor (Simpson et al 2000, Labrie et al 1998, Labrie et al 1997). Importantly, E2 synthesized within these sites is probably active only at a local tissue level, but the high concentrations achieved presumably exert significant biological influence *in loco* (Simpson et al 1999). Interestingly, among the myriads of stimuli that might contribute to induce autophagy under inflammatory conditions, sex steroids have recently been described as potential players (Yang et al 2013).

2. Objectives

I. Our previous light microscopical results showed that Freund's adjuvant treatment induces remarkable phenotypic changes in mesothelial cells. The first experiments of our studies were directed to certify in detail whether intraperitoneal Freund's adjuvant administration (induced inflammation) leads to epithelial-mesenchymal transition in mesenteric mesothelial cells *in vivo*. According to data from literature, TGF-β has an universal role in EMT. We were also interested in revealing whether the cytokine plays role upon inflammation in our system. The following questions were addressed:

1) What are the ultrastructural changes that can be observed in mesothelial cells upon the inflammation and the following regeneration?
2) Can inflammatory cytokines (IL-1, IL-6) be detected in the peritoneal fluid upon Freund's adjuvant treatment?
3) Does TGF-β have any roles in the inflammation-induced epithelial-mesenchymal transition? If so, how the level of its peritoneal secretion correlates with the inflammatory events?
4) Can the elements of the canonical TGF-β signaling pathway be detected in mesothelial cells? If so, what are the cellular compartments along which the signaling molecules (TβRII/Smad7) are accommodated in correlation with the inflammatory events?
5) Does caveolar internalization have a role in the dynamics of TGF-β signaling?

II. Recently, ER-α has been suggested as another player in the molecular mechanism of EMT. We previously demonstrated that mesothelial cells can assume a macrophage character by expressing ED1 (macrophage marker) and might be a source of peritoneal macrophages upon the inflammatory stimuli. Since macrophages are well-known to express estrogen-receptor α (ER-α), we were interested in whether the receptor might be present in mesothelial cells and has a role in TGF-β induced EMT. Based upon our previous results and the data from literature we considered to answer the following questions:

1) Do mesothelial cells express ER-α upon steady state and inflammatory conditions? If so, what is the subcellular distribution of the receptor?

2) Does the level of ER-α expression change during the inflammation?

3) Do ER-α and elements of the TGF-β pathway meet in any cytoplasmic compartments (caveolae, multivesicular bodies)?

4) Is there any natural ligand of ER-α in the peritoneal fluid and if so, what can be the role of estradiol during EMT and/or regeneration?

III. The detected and prolonged secretion of extragonadal estradiol raised the question about its possible role in tissue remodelling, regeneration. Among the several factors, sex steroids have recently been described to induce autophagy and help in tissue remodelling. Thus our interest turned towards examining whether possible estradiol-induced autophagy is present in mesothelial cells following acute inflammation and whether the process might contribute to the morphological re-establishment of the mesothelium. The following questions were addressed:

1) Is autophagy present and may play role in the removal of cytoplasmic organelles following inflammation? If so, how the rate of autophagy correlates with the inflammatory/regenerative events and the secreted E2 concentrations?

2) Does extragonadal estradiol have pivotal role in inducing autophagy? Are there any extracellular signals (TNFα) that are present and might aid or overwrite the effects of estradiol in our system?

3. Material&Methods

Material: Rat mesentery (peritoneum of small intestine) was removed from control and Freund's adjuvant (Sigma-Aldrich, Steinheim, Germany) treated Sprague-Dawley male rats (200–400 g). 1 ml complete Freund's adjuvant was injected into the peritoneal cavity. One day (D1), two days (D2), three (D3), five (D5), six (D6) eight (D8) and eleven (D11) days following intraperitoneal injections the animals were sacrificed by decapitation. Mesentery samples were further processed for morphological experiments and isolated mesothelial cells were used for the biochemical experiments.

Applied techniques and methods:
- single and double immunolabeling on semithin cryosections
- double immunolabeling on ultrathin cryosections (Tokuyashu-technique)
- conventional electron microscopy
- mesothelial cell isolation by collagenase digestion
- immunoprecipitation and Western blotting with whole cell lysates of isolated mesothelial cells
- measurement of mRNA expression levels with quantitative real-time polymerase chain reaction (qRT-PCR) from extracts of isolated mesothelial cells
- chemiluninescence immunoassay for determining estradiol hormone levels in plasma and peritoneal fluid samples

4. Results

4.1 The morphological and biochemical characterization of TGF-β induced EMT in mesothelial cells *in vivo*

4.1.1 Ultrastructural evidences of type II EMT in mesothelial cells *in vivo*

Our previous light microscopical data revealed that mesothelial cells lose their epithelial character (decreased cytokeratin and E-cadherin expression) upon Freund's adjuvant treatment and assume a mesenchymal phenotype by expressing vimentin (Katz et al. 2012). Upon inflammatory stimuli remarkable changes appeared in the ultrastructure of mesothelial cells as well. The dynamics of these changes showed a culmination on the second to third day (D2/D3) after treatment. From the fifth day (D5) on the tissue started to recover and the repairment was morphologically accomplished by the eleventh day (D11). Characteristic ultrastructural changes were the disintegration of basal membrane and transformation of the squamous mesothelial cells into individual cuboidal-shaped cells in two days after treatment. By the fifth day, the cells developed numerous lamellar processes and became spindle-shaped. The cytoplasmic compartments were more prominent: an increased number of mitochondria, polyribosomes, numerous vesicles and a growing number of multivesicular bodies (MVBs) could be observed in parallel with the inflammatory events of the surrounding tissue from D2. By D11 mesothelial cells retrieved their flat morphology and became integrated with the underlying connective tissue by the rearrangement of the basal lamina.

4.1.2 Inflammatory cytokines and TGF-β are released into the peritoneal cavity upon Freund's adjuvant treatment *in vivo*

It was of primary importance to see if Freund's adjuvant treatment induces inflammatory responses in our *in vivo* system at a molecular level as well. To verify this, we determined the expression levels of pro-inflammatory cytokines (IL1 and IL6)

in mesothelial cells as they are well-known to be involved in immune responses and inflammatory processes. The results of quantitative RT-PCR showed that mRNA expression levels of interleukin type 1alpha and type 1beta and also interleukin 6 increased in mesothelial cells in response to Freund's adjuvant treatment. The elevated mRNA levels of these cytokines correlated with the dynamics of the observed morphological changes during the inflammatory events: expression levels of mRNAs had a peak on D3 followed by a significant downregulation that could be observed from the fifth day indicating the termination of the inflammatory response. The Western blot data proved that TGF-β was secreted into the peritoneal cavity upon inflammation and showed a peak between D2 and D3.

4.1.3 The morphological detection and the subcellular distribution of the main canonical TGF- β signaling molecules in mesothelial cells

To map whether the elements of TGF-β signaling are present in mesothelial cells, we selected major downstream molecules (TβRII&Smad7) that are considered indispensable either in the promotion or in the termination of the signaling. Our confocal microscopical results showed that the receptor was expressed and located both along the plasma membrane as well as inside the cytoplasm. Under steady state conditions, intracellular receptor labeling appeared in early endosome antigen-1 (EEA1) positive compartments. By D3 the majority of the detected TβRII labeling occured inside the cytoplasm and was predominantly found in vesicular (punctate) structures overlapping substantially with EEA1. By D5 numerous large colocalization puncta could still be identified in the cytoplasm of mesothelial cells.

Since caveola-mediated internalization of TβRs presumably enhance the termination of TGF-β signaling, we investigated possible co-localization of TβRII and caveolin-1. The light microscopical results showed that in control mesothelial cells TβRII could not be detected together with caveolin-1. At the peak time of inflammation (D3), however TβRII abundantly appeared all over the cytoplasm of mesothelial cells and several overlapping puncta (indicating colocalization) could be observed between

the two markers. With the progression of inflammation, by D5 substantial cytosolic co-labeling between TβRII and caveolin-1 was present in mesothelial cells.

To obtain data about the presence and localization of negative regulatory protein, Smad7, we carried out double immunolabeling and found that on D3 samples the protein was expressed and could be observed both inside the cytoplasm as well as along the plasma membrane. Whenever we could detect Smad7 at the plasma membrane, it showed colocalization with caveolin-1.

4.1.4   En route to multivesicular bodies: the possible role of caveolar internalization in TGF-β signaling pathway

During inflammation related EMT, a notable increase in the number of cell organelles could be observed in mesothelial cells (mitochondira, endocytic vesicles). Multivesicular body (MVB) formation is a crucial compartment of the classical endocytic pathway for degrading internalized cargos. Fine structural and morphometric analysis affirmed that MVB formation was significantly increased at D3 in response to treatment and our immunoprecipitation results also showed that by this time, TβRII was co-immunoprecipitated with multivesicular body marker, Cd63. In contrast, this could not have been observed by D5 when TβRII was already not associated to Cd63.

Since there were reports demonstrating that after caveola-mediated endocytosis, the TβRs are degraded via lysosomal pathway, it was indicative to see at which point caveolae might meet the endo-lysosomal system. To obtain data, we carried out EEA1 and cavelin-1 double-labelled immunofluorescence assay. By D3, there were numerous orange (colocalization) dots underneath the plasma membrane of the cells indicating that the two markers - thus the compartments they label - meet at this time of the inflammation. The finer morphological results were consistent with the light microscopical data. By D3, we detected endosomes immunopositive for both EEA1 and caveolin-1 markers and these compartments were corresponded to forming MVBs. Our morphological data was further affirmed by the immunoprecipitation results: on D3 samples caveolin-1 was found to be associated with multivesicular body marker Cd63. In contrast, by D5 caveolin-1 was co-immunoprecipitated with rab7 antibody (marker of late endosomal trafficking downstream of MVBs). Our Western blot analysis also

revealed that caveolin-1 is entirely degraded at a protein level by D6 and only by D11 - with the morphological restitution of mesothelial cells - reached again detectable level.

4.2 The possible role of ER-α and estradiol in the process of EMT

4.2.1 Estrogen receptor alpha (ER-α) expression and its subcellular distribution in mesothelial cells

Our present results show that ER-α is present in mesothelial cells both under steady state and inflammatory conditions. Confocal microscopical results after immunofluorescence detection of ER-α showed that labeling appeared in the nucleus, cytoplasm as well as in the plasma membrane. On the consecutive days after treatment the intensity of the labeling increased. Distribution of ER-α labeling along the PM changed during the inflammatory process: while in control samples the labeling appeared all over the PM, it clustered at certain areas of the plasma membrane in treated cells.

4.2.2 The changes in the expression of ER-α upon inflammatory events

To further corroborate the presence of ER-α in mesothelial cells and how its expression changes upon inflammation induction, we measured it both at the mRNA and protein levels. The results of quantitative RT-PCR revealed a significant downregulation of ER-α mRNA expression in mesothelial cells during the progression of inflammation *in vivo*. (We found the same pattern of changes in the mRNA expression levels of ER-β and G protein-coupled receptor 30, GPR30 as well). However, inconsistent with the RT-PCR results, when we examined the receptor expression levels with Western blot, we found a significant increase of ER-α protein expression between D3 and D5. Further on, between D8 and D11 there was no detectable protein band.

4.2.3 The intersection of ER-α and TGF-β pathway at the level of caveolae

Our confocal microscopical results show that under inflammatory conditions ER-α colocalized with caveolin-1 both inside the cytoplasm and in the PM. There were no differences in the distribution of the two markers between D3 and D5. Consistent with the immunofluorescence data, the same distribution of ER-α and caveolin-1 could be found on double-labelled ultrathin frozen sections of treated cells. The immunoelectron microscopic results clearly showed that during the inflammation (D3 and D5) ER-α occurred not only at the plasma membrane but appeared also inside the cytoplasm. Here ER-α (together with caveolin-1) was localized in forming or mature MVBs either in their limiting membrane, in association with caveolin-1 or in caveolae, in close vicinity of MVBs. In control mesothelial cells ER-α labeling was accumulated along the plasma membrane on both the luminal and basolateral sides of the cells, preferentially in caveolin-positive vesicles, caveolae. Our morphological results predicted that ER-α and TGF-βRII might use the same internalization route from the peak time of inflammation, via caveolae. Our confocal microscopical results affirmed that indeed there is a remarkable co-labeling between ER-α and TβRII in the cytoplasm of mesothelial cells at D3.

4.2.4 Extragonadal estradiol (E2) is detected in the peritoneal cavity under *in vivo* inflammatory circumstances

Our present results show that under *in vivo* circumstances E2 could be detected in the peritoneal wash following Freund's adjuvant induced acute inflammation. E2 hormone concentrations significantly elevated by the third day (D3) in response to treatment and showed a prolonged secretion even after the attenuation of inflammation (between D5 and D11). In contrast, E2 hormone concentrations were unchanged and barely detectable in the plasma. These data indicated the presence of extragonadal E2 where biosynthesis is presumably achieved by aromatization of androgens to estradiol in adipocytes. By knowing that sex steriods may contribute to tissue remodelling via

autophagy induction, we considered that the prolonged secretion of E2 might play similar role in our system as well.

4.3 The role of autophagy in tissue remodelling

4.3.1 The role of autophagy in the retrieval of simple squamous morphology of mesothelial cells following acute inflammation

Using conventional electron microscopy, we observed autophagy even under control conditions but the number of AVs markedly rised five days after treatment. Quantitative morphometric analysis also affirmed that autophagy is mostly present at D5 in mesothelial cells. Microtubule-associated protein light chain 3 (LC3) is widely used and a well-validated biomarker of autophagy. Western blot results showed that conversion of LC3-I (inactive form) to LC3-II (active form) was remarkably higher and gradually increased between D5 and D8 indicating that indeed the formation of autophage vacuoles was present during the period of regeneration.

4.3.2 The possible inducers of autophagy (E2, TNF-α): extragonadal estradiol is in the focus

To assess/rule out the role of E2 in the induction of autophagy, we followed the expression pattern of TNF-α as well that is generally known to contribute to autophagy. The qRT-PCR results show that TNF-α mRNA levels were significantly elevated from D2 after inflammation induction and remained high between D5 and D11 as well (regeneration period). Estrogen induced autophagosome formation and maturation involves the activation of MAPK pathways such as Erk. Besides this, the involvement of extracellular signal-regulated kinase (Erk) 1/2 in the enhancement of TNF-α induced autophagy is well known. As Erk protein was found as a common mediator of both E2 and TNF-α induced autophagy, we measured the phosphorylation status of this protein in whole cell lysates of isolated mesothelial cells. Our Western blot results showed that

the active (phosphorylated) form of the protein was massively expressed at D6 (period of regeneration).

5. Conclusion

1) We established a type II, inflammation-related EMT model in which the cellular mechanism of the trasformation of mesothelial cells could be examined under *in vivo* circumstances.

2) Isolating mesothelial cells let us examine from a biochemical point of view the finer cellular changes occurred at the protein and mRNA levels upon inflammation and the following regeneration.

3) We examined the ultrastructural changes in mesothelial cells initiated upon Freund's adjuvant treatment and also proved the presence of inflammatory citokines and TGF-β in our system.

4) We affirmed the presence of TβRII and Smad7 proteins (elements of canonical TGF-β pathway) in mesothelial cells and our morphological observations and biochemical experiments proved that upon inflammation they internalized and reached the degradative pathway. In this process the caveolae and caveolin positive structures played important role.

5) Our novel finding was that ER-α is expressed in mesenteric mesothelial cells. Besides the commonly described nuclear and cytoplasmic localization of the protein, we found abundant receptor labeling in the plasma membrane of the cells as well. The receptor showed exclusive colocalization with the coat protein caveolin-1 both under steady state and inflammatory conditions.

6) Our present results showed that under *in vivo* inflammatory circumstances extragonadal E2 was secreted into the peritoneal cavity and is most likely contribute to the morphological reestablishment of the mesothelium by promoting autophagy.

7) From the peak time of inflammation and during the regeneration autophage vacuoles were abundantly present in mesothelial cells that was followed by the appearance of original simple squamous morphology.

6. List of Publications

List of publications related to the theme of this PhD:

1) Balogh P, Katz S, Kiss AL. (2013): The role of endocytic pathways in TGF-β signaling. Pathol Oncol Res, 19:141-148 (**IF:1,555**)

2) 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, (11) p. e79508. 10 p. (**IF: 3,73**)

3) 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 Research 18: 641-649, (**IF: 1,555**)

List of publications not related to the theme of this PhD:

1) Katz S, Balogh P, Kiss AL. (2011) Mesothelial cells can detach from the mesentery and differentiate into macrophage-like cells. APMIS, 119: 782-793 (**IF: 1, 991**)

7. Acknowledgement