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TGF-β1 promotes transition of mesothelial cells into fibroblast phenotype in response to peritoneal injury in a cell culture model

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ABSTRACT

Background: Peritoneal adhesions are a clinical problem. A key to the understanding of peritoneal adhesions is to study the healing of mesothelial cells within the peritoneal cavity following surgery. Transforming growth factor beta (TGF-βs) affects this healing process. The aim of this study was to investigate the effects of different concentrations of TGF-β1 on the healing rate and healing properties of mesothelial cells.

Materials and methods: Human mesothelial cells from peritoneal fluid were collected, cultured and a mechanical wound was created. The restoration of the mesothelial surface with and without increasing concentrations of TGF-β1 was monitored.

Results: The denuded area was restored within 24 h. The healing rate was most extensive between the first and second hour after the damage (61.9 ± 22.8 μm/h). No significant difference in healing rate were observed when increasing levels of TGF-β1 were used. However, higher concentrations of TGF-β1 increased cell size and the cells presented more fibroblast specific properties. Lower TGF-β1 concentrations increased the number of proliferating cells.

Conclusions: This study indicates the importance of high levels TGF-β1 in mesothelial cell healing, mainly by changing the actual healing properties of the cells. Elevated levels of TGF-β1 might promote mesothelial cell transition towards a more fibroblast-like appearance.

1. Introduction

Post-surgical adhesions are an important clinical problem. Trauma to the abdominal cavity, for example caused by abdominal surgery, is responsible for up to 90% of all adhesions.[1] The healing process of the peritoneal surface is a key component in adhesion formation.[2] The peritoneal surface is a basal membrane of connective tissue with a monolayer of mesothelial cells. These cells have the ability to regulate, produce and secrete several enzymes in the peritoneal environment, together with an exclusive ability to heal damage on the peritoneal surface. A restored peritoneal surface after surgical trauma is a prerequisite to restore the normal peritoneal environment[3] and interactions with surrounding cells.[4] Peritoneal surface repair differs from traditional wound healing. The size of the peritoneal defect is of lesser importance. Small and large peritoneal defects heal rapidly with similar healing rates using a simultaneous regeneration of the mesothelial surface without scar formation. The exact mechanism behind the restoration of the peritoneal surface is yet to be fully uncovered and to further explore peritoneal healing experimental models using both cultured omentally derived mesothelial cells[5] as well as murine animal models[6] have been used.

The role of transforming growth factor beta (TGF-β) in peritoneal restoration and repair of the peritoneal surface is assumed to be of importance although not fully elucidated. Human TGF-β exists in three isoforms (TGF-β1-3), all requiring activation to become biologically active.[7] They are major stimulators of matrix protein deposition known to induce fibrosis[8] and there is some evidence for their role in peritoneal repair through anti-fibrinolytic effects.[9] The presence of TGF-β1 in peritoneal tissue[10] and fluid[11] during surgery has been described. TGF-β1 has an anti-proliferative effect on mesothelial cells,[8,12,13] however, it has been shown that low concentrations of TGF-β1 increase the proliferation rate in human cultured peritoneal mesothelial cells,[11] thus suggesting simply eliminating TGF-β1 is not the way to reduce adhesion formation.

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However, reduction of the expression of TGF-β1, for example by reduced bleeding in the surgical field has been recommended as a way to minimize adhesion formation.[14] Other modifications of surgical technique can also affect the levels of TGF-β1, for example heated inflated carbon dioxide results in lower levels of active TGF-β1,[15] prolonged laparoscopic surgery may increase levels of TGF-β1[16] and the type of dissection device also affects levels of TGF-β1, where the use of an ultrasonic device decreases the levels of TGF-β1 compared to the use of electrocautery.[17] In order to recommend certain surgical adjustments to reduce adhesion formation the role of TGF-β1 in peritoneal surface repair requires further study as data indicate that both the levels and the timing of the doses of TGF-β1 are of importance.[9,14,18–20] The primary aim of this study was to evaluate the effect of increasing concentrations of TGF-β1 on the healing rate and healing properties of mesothelial cells.

2. Materials and methods

2.1. Ethical considerations

All patients gave written consent and the local ethics committee at the University of Gothenburg approved this study.

2.2. Mesothelial cell culture

Primary isolated human mesothelial cells collected from peritoneal fluid from patients undergoing surgery at Sahlgrenska University Hospital/Östra were cultured until the second passage as previously described.[21–23] Isolated peritoneal mesothelial cells resulted in established cultures that grew to confluence without any overlapping cells within one week. The recovered and reused media resulted in islands of mesothelial cells in 80% of the experiments after 24 h. Established cultures were seen within a few days (Fig. 1).

If not otherwise stated, all cell culture chemicals were purchased from Sigma–Aldrich (St Louis, Mo, USA). During routine culture, medium E199 supplemented with 30 IU/mL penicillin and streptomycin (PEST), 1.1 mM L-glutamin, 20% foetal calf serum (FCS), 0.5 μg/mL hydrocortisone, 50 μg/mL ECGF (prepared according to Maciag et al.)[25] and 10 IU/mL Heparin (Lövens, Sweden) was used.

The mesothelial origin was characterised by morphologic appearance and immunostaining.[21] Cells were sub-cultured on 12-well cell culture plates (Nunc, Roskilde, Denmark) with a cell density of 1×10^5 cells/well and maintained for 5–7 days until 80% confluent. Parallel cultures were stored in cryo tubes in liquid nitrogen for use in confirmatory tests. Prior to experimental start a pre-incubation for 24 h with medium containing 1% FCS only was initiated for all cells included in the experimental models of mesothelial injury.

As concentrated FCS could contain TGF-β1 and to confirm that the medium was contamination-free a commercially available ELISA (Promega Corporation, Madison, WI, USA) [Falk, 2008 #2947] was used to detect TGF-β1. Neither culture medium (with 20% FCS) nor dilution medium (with 1% FCS) showed detectable levels of TGF-β1.

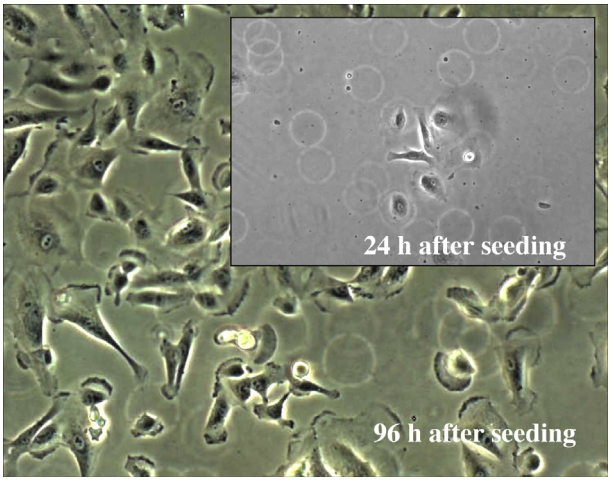

Fig. 1. Established mesothelial culture from free-floating mesothelial cells. Used culture media was recovered into new cell culture plates and resulted in established subconfluent mesothelial surfaces within a period of 96 h.

2.3. Experimental model of mesothelial injury

This model is a traditional mesothelial wound healing assay and a similar wound healing model using cultured fibroblasts have previously been described.[24] In brief cultured mesothelial cells were grown until confluence at experimental start. By scraping the monolayer of cells with a sterile plastic pipette (10 μL, D10ST, Gilson, Middleton, WI, USA) a mechanical injury was created (Fig. 2a). We used a plastic pipette to create the mechanical damage as we found that the previously described method using a glass-rod[5] easily damaged the plastic surface of the culture plate. The damage resulting from the pipette was measured with a calibrated size marker (Fig. 2b) using the Zeiss software (Axiovision, Carl Zeiss AG, Germany). The healing rate was calculated by dividing the restored distance with the time between each measurement.

2.4. Mesothelial injury and effects of TGF-β1

After the mesothelial surface was injured, cells were incubated with medium containing increasing concentrations (from 1×10^{-4} to 1×104 pg/mL) of activated (acidified) TGF-β1 (R&D, Abingdon, UK) in culture medium with 1% FCS. An equal number of plates with cells cultured with medium and 1% FCS served as controls. The mesothelial surfaces were monitored after 1, 2, 4, 6, 8, 24, 48, 72 and 96 h in order to follow the re-mesothelialisation. The cell sizes along the edge of the mechanical damage were calculated and documented using both the individual dimensions (height × width) of the cell and the tool in the axiocision software plotting a region of interest around the selected cell. For each concentration of TGF-β1, experiments were run in separate duplicates. Data is represented by four replicates in each experimental setup.

2.5. Morphology and immunostaining following TGF-β1 treatment

Cultured mesothelial cells were stained with a palette of antibodies to verify the mesothelial origin as previously described[21] and to ascertain that there was no contamination with other cells. In brief, cultured cells were fixed in 95% ethanol for 5 min. Following a PBS wash for 5 min, cells were pre-incubated for 5 min with 5% dried fat-free milk (Semper AB, Stockholm, Sweden) in PBS. Each incubation step was preceded using a PBS wash three times.

The following antibodies were used; mouse anti-human Cytokeratin 4.62 (Sigma–Aldrich) diluted 1:20, mouse anti-human Cytokeratin 19 (Dako A/S, Glostrup, Denmark) diluted 1:40, rabbit anti-human Fibroblast (Dako A/S, Glostrup, Denmark) diluted 1:50, rabbit anti-human von Willebrand factor (Daco A/S,

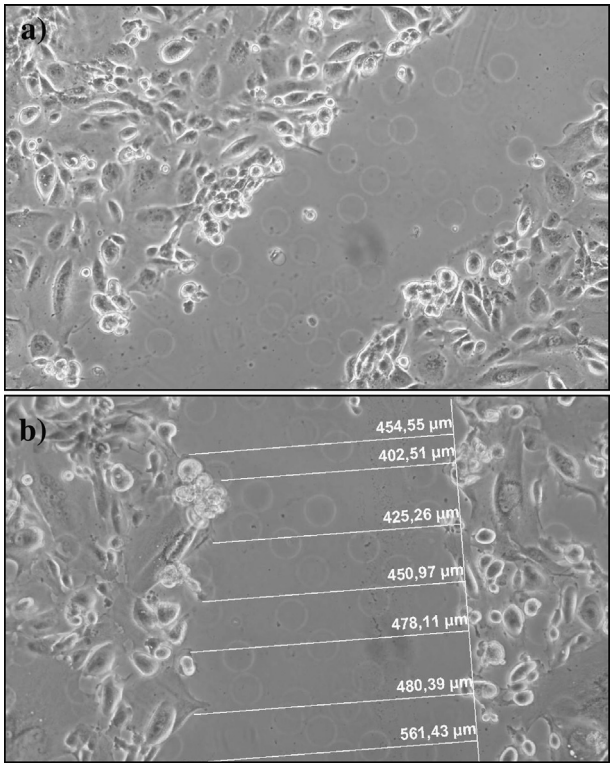

Fig. 2. A small sterile pipette tip, damaging the cultured mesothelial surface, created the experimental model of the mesothelial injury (a). The healing rate was then monitored and calculated by monitoring the remesothelialisation (b).

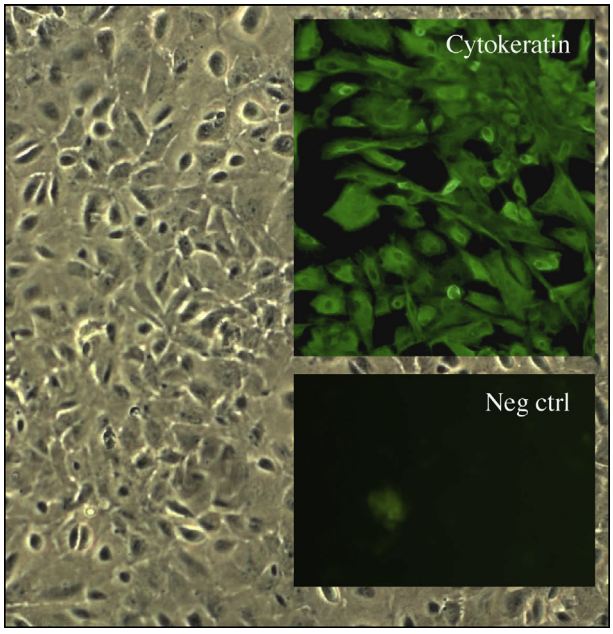

Fig. 3. Cultured human mesothelial cells with typical monolayer growth pattern, together with positive immunostaining for human cytokeratin.

Glostrup, Denmark) mouse anti-human Fibroblast (Dianova, Germany). The secondary antibodies used were FITC (Fluoroscens isothiocyanate) conjugated against mouse or rabbit (Dako) depending on primary antibodies used. Control serum from mouse and rabbit, with the same protein concentration as the primary antibody, were used as negative controls (Dako). All dilutions were made in PBS-buffer containing 0.5% bovine serum albumin (Sigma–aldrich).

The FITC labelling was detected using UV light connected to the inverted microscope.

Morphological appearance together with cytokeratin positive cells and absence of staining for fibroblast and endothelial specific properties indicates mesothelial origin (Fig. 3).

2.6. Statistics

The Kruskal–Wallis test was used to detect overall differences in healing rates and if significant, a Bonferroni corrected Mann–Whitney *U* test was performed between individual groups. A non-corrected *p*-value <0.05 were considered statistically significant and when all groups were compared a corrected significance level of <0.01 was used. When healing rate and mesothelial restoration were correlated with time the Spearman Rank Correlation was used. Results are presented as mean ± standard deviation (SD) in the figures. All statistical calculations were performed using the StatView 5.0 package (Abacus Concepts, Berkeley, CA, USA).

3. Results

3.1. Experimental healing rate after mesothelial injury

Mechanical damage resulted in a denuded injury with an average distance of 409.7 ± 38.7 μm (Fig. 2b). The injury was completely restored after 24 h (Fig. 4a). Healing rate was most rapid between the first and second hour after damage; 61.9 ± 22.8 μm/h (Fig. 4b). Both healing rate and mesothelial injury restoration were correlated with time, for healing rate ($r2 = -0.351$, $p = 0.025$) and mesothelial injury restoration ($r2 = 0.978$, $p < 0.0001$). Mesothelial injury restoration reached 90% confluence within 8–10 h (Fig. 4c).

3.2. TGF-β1 effects on mesothelial healing rate and healing properties

No difference in healing rate was seen between the different TGF-β1 concentrations compared to control medium. However, after 24 h mesothelial cells treated with higher concentrations of TGF-β1 healed the injured area by increasing in cell size with 12800 ± 6700 μm^2 ($p < 0.001$, $n = 15$) while cells with lower concentrations of TGF-β1 seemed to heal with an increase in number with 2900 ± 2400 μm^2 ($p = 0.217$, $n = 15$), compared to 3800 ± 2500 μm^2 for untreated medium control. This was also reflected in cell counts where cells treated with lower concentrations of TGF-β1 healed by an increase in number, with 56000 ± 17100 cells/cm^2 compared to 11000 ± 6800 cells/cm^2 for high concentrations of TGF-β1 compared to 36000 ± 18500 cells/cm^2 for the untreated control. Additionally, 72 and 96 h after mechanical injury mesothelial cells treated with higher concentrations of TGF-β1 developed a more elongated cell type similar to fibroblasts with a higher number of multi-nuclear cells. This effect could be seen after 72 h and sustained after 96 h of treatment with more fibroblast-like appearance and multi-layer growth pattern in a dose-dependent manner (Fig. 5).

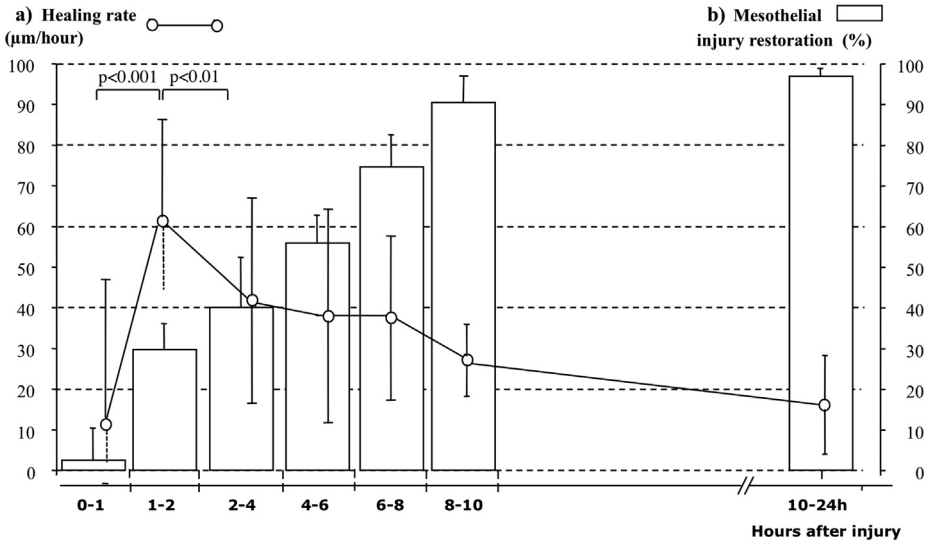

Fig. 4. Healing rate and mesothelial recovery after mechanical damage on the mesothelial surface after 24 h. The line graph (Mean ± SD) indicates lower healing rates immediately after the mechanical damage, followed by an increase within 1–2 h and then a decrease until the mesothelial damage was recovered (a: Left *Y*-axis). The mesothelial injury restoration during time is described using bar graphs (b: Right *Y*-axis, Mean ± SD).

Higher concentrations of TGF-β1 induced cells to stain for fibroblast specific antibodies as well as antibodies for mesothelial origin. Specific growth patterns with fibroblast like cells, growing in several layers, were also present in contrast to cells with low or no TGF-β1 (Figs. 5 and 6).

4. Discussion

This study increases the knowledge regarding peritoneal healing, which in the future may allow surgical techniques to adjust accordingly to reduce adhesion formation. The aim was to explore the role of different concentrations of TGF-β1 on peritoneal healing as it has previously been reported that the proliferation rate of mesothelial cells decreases with higher concentrations of TGF-β1[12,25] and that the proliferation rate increases with low concentrations similar to levels found in the human abdominal cavity during surgery.[11] Unexpectedly we found no relationship between proliferation rate and healing rate, but our results indicate that healing rate may not be the most important measure; perhaps the size and number of cells are more important. Cells with higher concentrations of TGF-β1 healed the injured area by increasing in size, while cells treated with lower concentrations of TGF-β1 seemed to heal by an increase in number. The latter result may partly explain our previous findings.[11]

Our data also suggest that higher levels of TGF-β1 for a long time affect the healing properties of the peritoneum other than by decreasing healing rate, as the mesothelial cells changed their morphology into a fibroblast like appearance. Mesothelial cells may

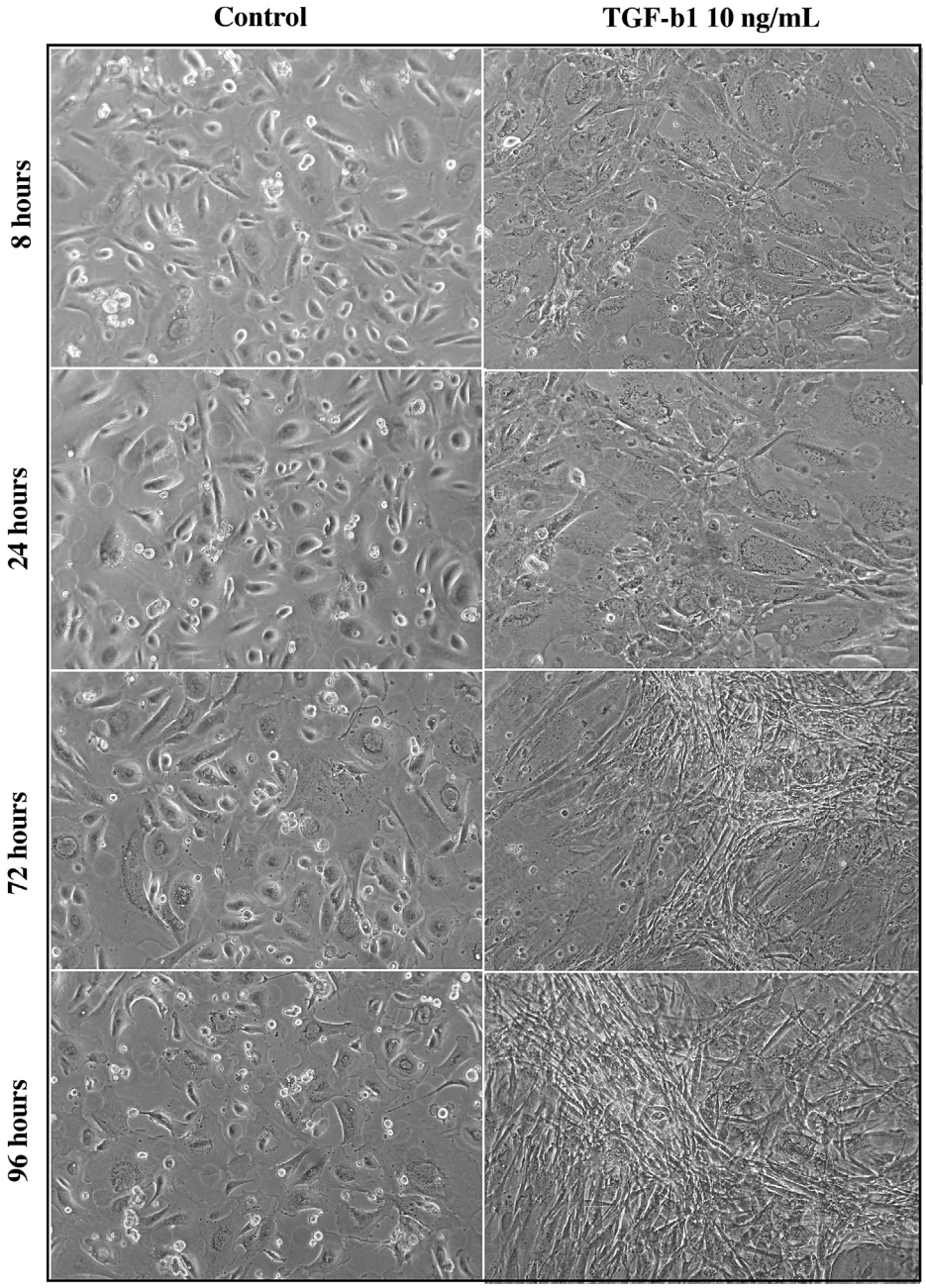

Fig. 5. Micrographs of the mesothelial surface when time and concentrations of TGF-β1 were compared. Mesothelial cells with control medium (left column) compared with higher (right column) concentrations of TGF-β1. Increasing time and concentration seemed to have effect on the mesothelial morphology in the cultures, turning the cells into a more fibroblast like appearance, with a multi-layer growth pattern.

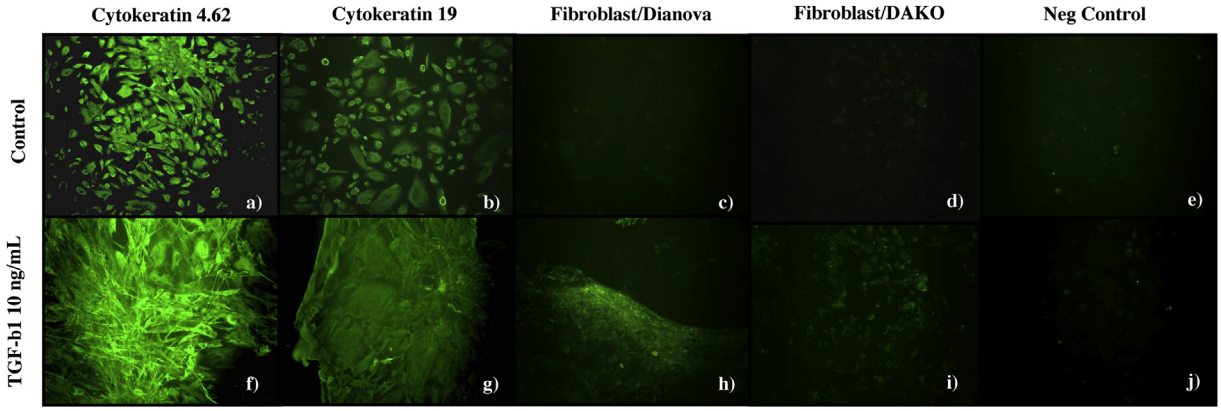

Fig. 6. Immunostaining of mesothelial cells using fluorescence isothiocyanate (FITC) and a palette of anti-human antibodies. Micrographs indicating a mesothelial origin in cells treated with no TGF-β1 by positive staining for cytokeratin and negative for fibroblast (Fig. 6a–e). Interestingly, with higher concentrations of TGF-β1 the positive staining for cytokeratin sustained together with a positive staining for fibroblast specific antigen (6f–j).

be a source of myofibroblast in the peritoneal cavity and our findings indicate that increased levels of TGF-β1 stimulates this transition. It is known that TGF-β promotes epithelial – mesenchymal – transition (EMT),[26] and our findings are most probably reflect this action by TGF-β1. The transition to fibroblast like appearance again indicates that TGF-β1 has the capacity to affect the healing process by fibrosis, which also increases adhesion formation.

The healing rate was highest within the first hour, which likely is due to a gradual decreased degree of healing as the cells cover the damaged surface. However, after 24 h the damaged area was restored which is in contrast to the study performed by Yung et al.[5] where the injury was healed after 72 h. In the current study we used primary isolated mesothelial cells from peritoneal fluid. It might be that mesothelial cells derived from peritoneal fluid and from the omentum react differently to mechanical injury, which could explain the difference in healing rate between our present study and the study performed by Yung.[5]

Similar to what has been described by Mutsaers et al.[27] free-floating cells that in routine cell culture, in vitro, are discarded together with the used culture media could form new established cultures of cells. In 4 out of 5 independent experiments cells were present and had the ability to form new cultures that increased in number and formed established cultures within a short period of time. This might be an important mechanism in the peritoneal environment for the restoration of mesothelial surfaces after damage.

Taken together, this study suggests a role for TGF-β1 in mesothelial cell recovery as it affects the mesothelial cell recovery through morphology changes rather than only proliferation rate. Future studies should continue to focus on the effects of high levels of TGF-β1 on mesothelial cell regeneration as reduction of TGF-β1 levels may decrease clinically significant adhesions.

Ethical approval

All patients gave written consent and the local ethics committee at the University of Gothenburg approved this study 2003-12-01 Ö727-03.

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Author contribution

Peter Falk, Eva Angenete, Maria Bergström, and Marie-Lois Ivarsson all contributed to study design. Maria Bergström, and Marie-Lois Ivarsson contributed to collection of cells. Peter Falk performed the experiments and the cell cultures, Peter Falk, Eva Angenete and Marie-Lois Ivarsson performed the data analysis. Peter Falk and Eva Angenete performed most of the writing of the first draft and preparation of the manuscript, but all authors participated in the writing process and critically revised it. Peter Falk, Eva Angenete, Maria Bergström and Marie-Lois Ivarsson approved the final version of this manuscript.

Conflict of interest

None.

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