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Intraabdominal adhesion formation is associated with differential mRNA expression of metabolic genes PDHb and SDHa

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Abstract
Purpose Intraabdominal adhesions represent a major cause of postoperative morbidity potentially causing pain, small bowl obstruction and infertility. The process of adhesion formation might be regarded as an ischemic disease. Under hypoxic conditions, metabolic enzymes are regulated via hypoxic responsive elements by the hypoxia-inducible factor 1 (HIF-1). We therefore investigated the gene expression of HIF-1 and two pivotal metabolic genes, pyruvate-dehydrogenaseβ (PDHb) and succinate-dehydrogenase-complex-subunit-A (SDHa) in a validated ischemia model of adhesion formation.
Methods Peritoneal adhesions were created using an ischemic button model in female Wistar rats. Expression levels of HIF-1α/β, PDHb and SDHa in adhesiogenic versus native peritoneum were analyzed using quantitative PCR on the third post-operative day.
Results Gene expression of HIF-1α was up-regulated by 10 % ($p = 0.003$), PDHb was up-regulated by 23 % ($p = 0.0004$) and SDHa ($p = 0.0005$) was up-regulated by 24 % in the adhesiogenic peritoneum compared to native peritoneum. The expression level of HIF-1β was not significantly influenced by adhesion formation.
Conclusion The increased expression level of HIF-1α in the peritoneal tissue of ischemic buttons associated with postsurgical adhesions supports the major role for hypoxia in influencing peritoneal expression patterns of genes involved in the process of adhesion formation. As pivotal metabolic genes are upregulated, this seems to be an anabolic process associated with increased cellular metabolism.

Keywords Peritoneal adhesion · Ischemia · Hypoxia inducible factor · Metabolic genes · Rat animal model · Button model

Introduction

Peritoneal adhesions represent a major cause of postoperative morbidity potentially causing chronic pelvic pain, intestinal obstruction, female infertility and difficulties at the time of reoperation [1]. In the pathological process of adhesion formation, ischemia is known to play a pivotal role. Most mammalian cells can respond to oxygen level alterations by increasing or decreasing the expression of specific genes [2]. The hypoxic regulation of many of these genes, such as plasminogen activator inhibitor (PAI) and vascular endothelial growth factor (VEGF), takes place at both, transcriptional and posttranscriptional levels. The transcriptional regulation is mediated by transcription factors known as hypoxia inducible factors (HIFs) [3]. HIFs are nuclear proteins that bind to hypoxia response elements (HRE) in the promoter or enhancer regions of hypoxia inducible genes, activating gene transcription in

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response to reduced cellular oxygen levels [4]. They are the members of the basic helix-loop-helix (bHLH) periodic (Per) aryl hydrocarbon receptor nuclear translocator (ARNT) single-minded domain protein family (PAS) [5]. Several proteins have been identified in this bHLH-PAS family that belong to the α-class or the β-class. Each member of the α-class is able to heterodimerize with a member of the β-class to form a stable activation complex. β-class members are constitutively expressed in an ubiquitous or a tissue-specific way, whereas α-class members are often inducible by environmental stimuli such as hypoxia [6]. HIF-1 is a heterodimer composed of HIF-1α and HIF-1β subunits. HIF-2 is a heterodimer composed of HIF-2α and HIF-1β subunits. HIF-1α and HIF-2α, the specific hypoxia-regulated subunits, are structurally very similar and share the same heterodimerization partner. Therefore, both HIF-1 and HIF-2 have a high similarity in structure and regulatory domains and are able to bind to the same HRE of target genes [5].

The role of HIFs in adhesion formation has been recently demonstrated in animal models. Indeed, down-regulation of HIF-1 reduces adhesion formation in rats [7], whereas pneumoperitoneum-enhanced adhesion formation is absent in mice partially deficient for HIF-1α and HIF-2α [8].

As HIF-1 plays a pivotal role in the maintenance of O_2 homeostasis, we concluded that the process of adhesion formation might, at least in part, be regarded as an ischemic disease. Under anaerobic conditions, when O_2 is unavailable as the final electron acceptor in the respiratory chain, the cell must abandon oxidative phosphorylation and rely solely on glycolysis for energy production. Induction of almost all glycolytic enzymes is dependent on HIF-1 [9]. We therefore evaluated the expression of HIF-1 α/β in a validated ischemia in vivo model of adhesiogenesis. We further hypothesized that cell metabolism might be altered. To prove this hypothesis, we analyzed expression levels of two pivotal metabolic genes, pyruvate-dehydrogenaseβ (PDHb) and succinate-dehydrogenase-complex-subunit-A (SDHa).

Methods

Animals

Three female Wistar rats with a weight range of 225–270 g (Charles River Laboratory, Sulzfeld, Germany) were used. Rats were housed under laboratory conditions in the animal facility with food and water available ad libitum.

Surgeries

Abdominal adhesions were created using a validated model [10–12]. Briefly, after mid-line laparotomy three 5-mm buttons of parietal peritoneum were grasped with a hemostat and the base was ligated with a 4-0 Prolene suture (Fig. 1). The buttons were placed unilaterally. The laparotomy incision was closed in two layers using continuous 3-0 Vicryl suture.

After 3 days, six tissue samples from each animal were harvested, three from the tip of each ischemic button (peritoneal adhesion sites) and three from the contralateral native peritoneum (control sites). Corresponding with the peritoneal adhesion sites, the native peritoneum was grasped with a hemostat and excised at the tip of three created non-ischemic buttons with a scalpel.

Quantitative PCR

Total RNA was isolated from the peritoneal tissue of the buttons and from the contralateral native peritoneum with the RNeasy Plus Mini Kit (QIAGEN) and cDNA was generated using SuperScript III RNase H-Reverse Transcriptase (Invitrogen). Gene expression was quantified with the TaqMan Gene Expression Assay (Applied Biosystems). Results were standardized with the expression of beta-actin mRNA and given as relative expression to TACR1, which is constitutively expressed and an established control gene for adhesion studies [12, 13]. Specific primers used for quantitative RT-PCR are: HIF-1α (forward primer: TGG ATGGCTTTGTTATGGTG; reverse primer: TGGTCA CATGGATGGGTAAA), HIF-1β (forward primer: CATGT CTCTTCCGGGTGCT; reverse primer: GTCCGGG TCTGGAACTGTC), PDHb (forward primer: GTAGAGGA CACGGGCAAGAT; reverse primer: TTCACGAAC TGTCAACTGCAC) and SDHa (forward primer: TGG ACCTTGTCGTCTTTGG; reverse primer: TTTGCCTT AATCGGAGGAAC).

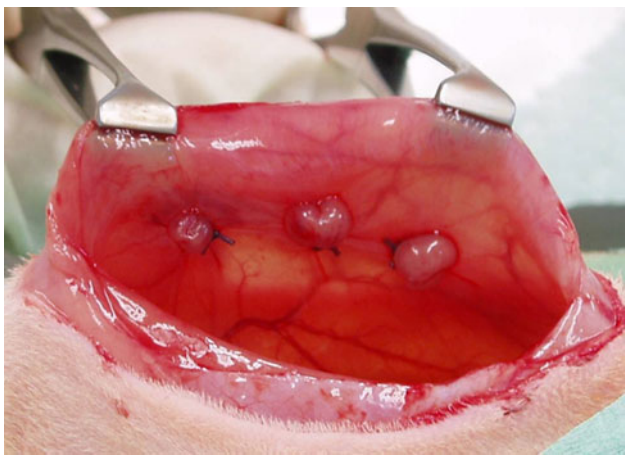

Fig. 1 Peritoneal button model in female Wistar rats. After laparotomy a 5-mm button of parietal peritoneum was grasped and the base was ligated with a 4-0 Prolene suture

Experimental design

Adhesions were induced in all rats according to an established model [10–12]. The differential expression level of HIF-1α, HIF-1β and the metabolic genes, PDHb and SDHa, was determined at post-operative day 3 in the peritoneal adhesions sites and compared with the control sites.

Statistical analysis

Statistical analysis was conducted using JMP (Version 7; SAS Institute Inc., Cary, NC, USA). The average gene expression level within the three buttons of an animal was compared to the average gene expression level within the three probes of native peritoneum of the same animal by the use of Student's two-tailed paired t test. The significance level was set to $p < 0.05$.

Results

Adhesions were detected in all nine (3×3) lesion sites. Quantitative PCR demonstrated a significant up-regulation by 10 % of HIF-1α expression in the ischemic button as compared to native peritoneum on the contralateral non-traumatised side (Fig. 2, $p = 0.003$). Gene expression of HIF-1β did not significantly change ($p = 0.09$). Expression of metabolic genes was also found to be significantly increased (Fig. 3): PDHb was up-regulated by 23 % ($p = 0.0004$) and SDHa was up-regulated by 24 % ($p = 0.0005$).

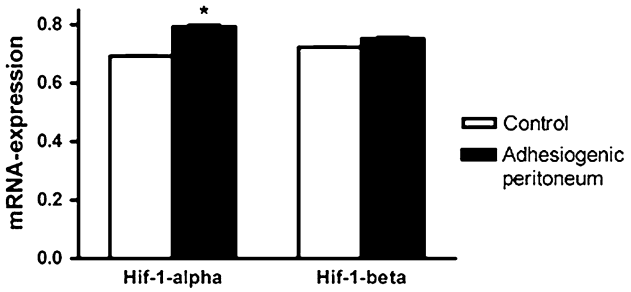

Fig. 2 Expression levels of HIF-1 in the control versus adhesiogenic peritoneum. Abdominal adhesions were created using an animal peritoneal button model. At day 3 post operation, total RNA was isolated from peritoneal tissue of the buttons and from contralateral native peritoneum. Results are given as expression level of HIF-1α/β relative to ACTB. Each *bar graph* represents the average expression of nine probes (three animals and three probes each animal) for adhesion tissue or control peritoneum. *Asterisk* indicates significant ($p < 0.05$) changes in mRNA levels as revealed by the paired Student's t test. *Error bars* indicate standard deviation

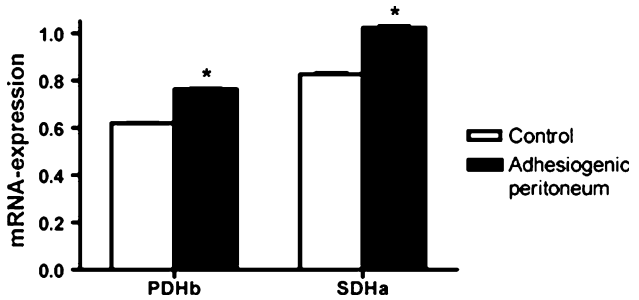

Fig. 3 Expression levels of metabolic genes PDHb and SDHa in the control versus adhesiogenic peritoneum. Abdominal adhesions were created using an animal peritoneal button model. At day 3 post operation, total RNA was isolated from peritoneal tissue of the buttons and from contralateral native peritoneum. Results are given as expression level of PDHb/SDHa relative to ACTB. Each *bar graph* represents the average expression of nine probes (three animals and three probes each animal) for adhesion tissue or control peritoneum. *Asterisk* indicates significant ($p < 0.05$) changes in mRNA levels as revealed by the paired Student's t test. *Error bars* indicate standard deviation

Discussion

Adhesions result from a cascade of molecular events that are initiated by surgical trauma and postoperative ischemia [14]. We therefore concluded that the process of adhesion formation might, at least in part, be regarded as an ischemic disease, where cell metabolism relies on glycolysis for energy production. We thus analyzed the expression of HIF-1, regulating almost all glycolytic enzymes and hypothesized that cell metabolism might be altered. To prove this hypothesis, we further analyzed the expression levels of two pivotal metabolic genes, pyruvate-dehydrogenaseβ (PDHb) and succinate-dehydrogenase-complex-subunit-A (SDHa). We used an ischemic button model that is among the most established models for adhesion induction [15] and chose day 3 for mRNA expression analysis when adhesion formation is in progress but not yet completed [14]. How differences in mRNA expression during adhesiogenesis are regulated or whether inhibition or activation of SDHa or PDHb prevents adhesion formation was beyond the scope of this study. A causative link between adhesiogenesis and altered expression of metabolic genes during adhesion formation remains to be established.

We found HIF-1α to be significantly up-regulated in adhesiogenic peritoneum when compared to control peritoneum. This is in line with previous reports, revealing that inhibition of HIF-1 via siRNA targeting reduces postoperative adhesions in rats [7]. As HIF-1β is constitutively expressed, we found no differences between native and adhesiogenic peritoneum. Interestingly, PDHb as well as SDHa, which catalyses an important step of the Krebs Cycle, was also up-regulated. These findings indicate an increased cellular metabolism within the peritoneal tissue during the process of adhesion formation.

The up-regulation of HIF-1α is likely to be induced by peritoneal ischemia leading to insufficient fibrinolysis and the attachment of traumatised areas to proximal tissues [16, 17]. The subsequent processes involved in organisation and remesothelization of adhesion tissue are mostly energy-dependent anabolic events. Persistent fibrin is invaded by polymorph-nuclear leucocytes (PMNs), monocytes and fibroblasts [14, 18]. When compared to primary cultures of fibroblasts from normal peritoneum, adhesion fibroblasts were shown to have higher proliferation rates [19, 20]. The fibrin matrix is subsequently organized by cellular structures, containing blood vessels and nerve fibers [21]. This explains an increased metabolism and the up-regulation of metabolic genes, PDHb and SDHa. As the observed adhesions were found to be highly vascularized, ischemia might further trigger genetic changes within mesothelial cells to actively attract adhesions and thereby provide a vascular graft to revascularize ischemic tissue [22]. In this context, HIF-1 is known to up-regulate VEGF [8]. As the expression analysis was performed at RNA-level only and HIF-1α is known to be regulated post-transcriptional (e.g., stabilized by hsp90; degraded by the ubiquitin–proteasome system) [23], it is possible that the increase in HIF-1α could be even more pronounced on protein level.

In conclusion, we found that the elementary metabolic processes are involved in the pathogenesis of adhesion formation. The organisation and remesothelization of adhesion tissue seem to be energy-dependent anabolic processes, indicated by an increased metabolism within the peritoneal tissue during adhesion formation. The HIF-1 seems to play a pivotal role in the underlying regulative processes and future studies should especially focus on proteins and cellular functions regulated by this factor. Moreover, this work encourages further investigations on the role of metabolic genes and their regulation during adhesion formation (e.g., to analyze mitochondrial involvement) and whether pharmacological alteration of metabolic pathways might be an approach to reduce postoperative adhesion development.

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Conflict of interest None to declare.

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