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Reactive Oxygen Species and EGR-1 Gene Expression in Surgical Postoperative Peritoneal Adhesions

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Abstract. Postoperative peritoneal adhesions are common and serious complications of general abdominal and gynecological surgery that can lead to chronic abdominal pain, small-bowel obstruction and infertility. The specific pathophysiology of peritoneal adhesions remains elusive and current treatment is relegated to prevention through meticulous surgical technique and protective physical barriers, gels and solutions. We have reported that reactive oxygen species (ROS), generated by phagocytic cells at the site of tissue injury, serve as major signaling molecules regulating the expression of vascular endothelial growth factor (VEGF) and subsequent wound repair. We hypothesized that peritoneal adhesions are a product of over-healing surgical wounds and that, like in wound healing, ROS are implicated in their pathogenesis. We examined the presence of footprints of ROS and the ROS-inducible angiogenic factor VEGF in human adhesion tissue. An experimental model of peritoneal adhesion was established in rodents to study of the dynamics of ROS-induced gene expression during de novo adhesion tissue formation. Immunohistochemical analysis demonstrated presence of ROS/oxidant and macrophages in human peritoneal tissue. The presence of ROS and ROS-sensitive transcription factor EGR-1 was also evident in an experimental rodent peritoneal adhesion model. Along with ROS, VEGF, and a large number of mature and immature CD31/vWF positive blood vessels were present in the adhesion tissue. These observations are not consistent with the contention that adhesions are non-functional scar tissue. The newly developed rodent model of adhesion may present a useful approach to reproducibly and objectively study molecular mechanisms underlying the dynamic process of de novo adhesion tissue formation.

Postoperative peritoneal adhesions are common and cause serious complications of general abdominal and gynecological surgery. Over 90% of the patients that undergo major abdominal surgery develop peritoneal adhesions [1, 2]. The complications caused by adhesions vary, but they include early postsurgical complications, development of chronic abdominal pain, late small bowel obstruction, and infertility in women [1, 2]. Although the problem is substantial, the specific pathophysiology of peritoneal adhesions remains elusive. As a result, current preventive measures are relegated to meticulous surgical technique and numerous protective physical barriers, gels, and solutions [3, 4].

Surgical trauma is one of the major triggers of postoperative adhesion formation. It is generally believed that adhesions are the end-result of aberrant peritoneal healing. Previously, we have observed that reactive oxygen species (ROS) generated by phagocytic cells at the site of tissue injury serve as major signaling molecules that regulate wound angiogenesis [5–7]. In the current work, we tested the hypothesis that peritoneal adhesions are a product of over-healing surgical wounds and that, as in wound healing, ROS are implicated in the pathogenesis. Thus, we sought to examine the presence of footprints of ROS and angiogenesis in human adhesion tissue. Furthermore, an experimental model of peritoneal adhesion was established in rodents to investigate the dynamics of ROS-induced gene expression during de novo adhesion tissue formation.

Materials and Methods

Human Peritoneal Adhesions

Peritoneal adhesion tissues were collected from patients undergoing major abdominal surgery. Samples were immediately fixed in formaldehyde and immunohistochemistry was performed to visualize the presence of phagocytic cells, blood vessels, and footprints of ROS. Ethical approval for this study was obtained from the Biomedical Sciences Institutional Review Board, The Ohio State University Medical Center, Columbus, Ohio.

Immunocytochemistry

Formalin-fixed tissues were embedded in paraffin and sectioned (4 μm). The sections were stained with the following primary antibody and appropriate secondary antibodies: CD68 (1:100; DakoCytomation), HAM56 (1:50; Novus Biologicals), nitrotyrosine (1:400; Upstate Biotechnology), vascular endothelial growth factor (1:100; R&D Systems Inc), CD31 (1:20; DakoCytomation), and von Willebrand (1:500; DakoCytomation). Immunoreactivity was detected

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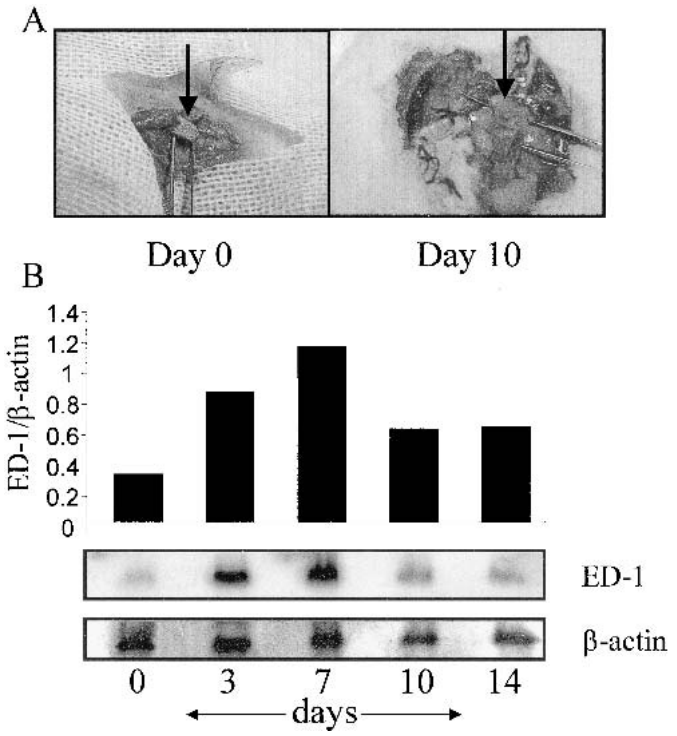

Fig. 1. De novo tissue formation and macrophage infiltration in an experimental model of peritoneal adhesion. A rat model of peritoneal adhesion was developed. The model employs a foreign material (Marlex) and trauma to induce adhesion tissue formation in the lower abdomen. **A**. For imaging, rats were euthansized and the peritoneal adhesion was imaged in situ with a digital camera (Mavica FD91, Sony). **B.** Western blot analysis was performed using ED-1 antibody that is know to recognize cells of the monocyte-macrophage lineage in rats. The blots were re-probed with β-actin to show comparable loading. **B,** top: For quantification, densitometry was performed. ED-1 expression is in arbitrary units and data are normalized to respective amount of β-actin protein.

using the avidin-biotin/peroxidase reaction (ABC kit, Vector Laboratories) and visualized by diaminobenzidine tetrahydrochloride (DAB) substrate. Controls were prepared by substituting the appropriate normal serum for the primary antibody.

Experimental Model

A rat model of experimental peritoneal adhesion was developed as described in the paragraphs that follow.

Survival Surgery. Rats ($n = 15$) were anaesthetized and their abdomen was opened through a midline incision. A small (1 × 1 cm) Marlex screen was sutured to the descending colon with two stitches of non-absorbable 6/0 monofilament nylon (Fig. 1). The peritoneum and skin were closed with running 4/0 Dexon sutures as two separate layers. After surgery, the rats were allowed to heal.

Imaging and Tissue Collection. On postoperative days 3, 7, 10, and 14, three rats were euthanized. The peritoneal adhesion was imaged in situ with a digital camera (Mavica FD91, Sony) and adhesion tissues were collected in liquid nitrogen for biochemical analysis and stored at –70°C.

mRNA Quantitation. EGR-1 mRNA was quantified with the Taqman real-time polymerase chain reaction (PCR) assay. RNA extraction and real-time assay were performed as described previously [8]. The following primers/probes were designed with Primer Express (Perkin-Elmer, Applied Biosystems) software for detection of rat EGR-1 gene: *Primer set*—5′ GCCTCGTGAGCATGACCAA; 5′ GCAGAGGAAGACGATGAAGCA. *Taqman Probe*—CGACCTCTTCATCCTCGGCGCC. Ribosomal RNA was used as an endogenous control. For quantification, the delta delta Ct method that normalizes to a housekeeping gene was used [9].

Western Blot. Western blot analysis was performed as described previously [10]. Primary antibodies against ED-1 (1:1000 dilution; Serotec) and EGR-1 (1:200 dilution; Santa Cruz) were used to detect macrophages and EGR-1, respectively.

Results

Immunohistochemical analysis of human adhesion tissue demonstrated a large number of macrophages (CD68 & HAM56 positive), indicating an ongoing acute/chronic inflammatory process (Fig. 2). Activated macrophages are known to simultaneously produce nitric oxide (NO) and superoxide (a ROS), two radicals that can react to form a powerful oxidant, peroxynitrite [11]. Peroxynitrite reacts with tissue protein tyrosine to generate nitrotyrosine. We investigated whether adhesion tissues show presence of nitrotyrosine, a footprint of peroxynitrite. The sections from adhesion tissue stained intensely positive for nitrotyrosine, confirming the presence of peroxynitrite, a product of macrophage oxidative metabolism. Macrophages and ROS/oxidants are known to induce angiogenesis. In the adhesion tissues studied, angiogenesis was evident by the presence of vascular endothelial growth factor (VEGF; Fig. 2). CD31 and von Willebrand factor (vWF) staining were performed to identify any endothelial cells lining the blood vessels and capillaries. All the adhesions examined were well vascularized, containing a large number of blood vessels (Fig. 2).

A rat model of experimental peritoneal adhesion was developed (Fig. 1A). The model employs a foreign material (Marlex Mesh; used in hernia operations) and surgical trauma to induce adhesion tissue formation in the lower abdomen. These two factors are known triggers of adhesion tissue formation in humans. As illustrated in Figure 1A, by day 10 the Marlex material was completely covered by de novo formed tissue. This approach was used to study the dynamics of adhesion tissue formation in the context of macrophage infiltration and footprints of ROS/oxidant-mediated cell signaling. Western blot was performed with antibody against ED-1, which is known to recognize cells of the monocyte–macrophage lineage in rats. The data showed that there was an initial surge of macrophages in the adhesion tissue between postoperative days 3 and 5, followed by inhibition of the rate of infiltration by such cells. Early growth response-1 (EGR-1), a zinc-finger transcription factor and member of the immediate early response gene family, is ROS-inducible [12]. This gene is also implicated in the regulation of angiogenesis and wound healing [13]. The kinetics of EGR-1 protein and mRNA expression was assessed in rat adhesion tissue as a marker of ROS/oxidant-mediated gene expression. Western blot analysis of EGR-1 protein (Fig. 3A) and quantitative mRNA expression detected by real-time PCR (Fig. 3B) indicated that

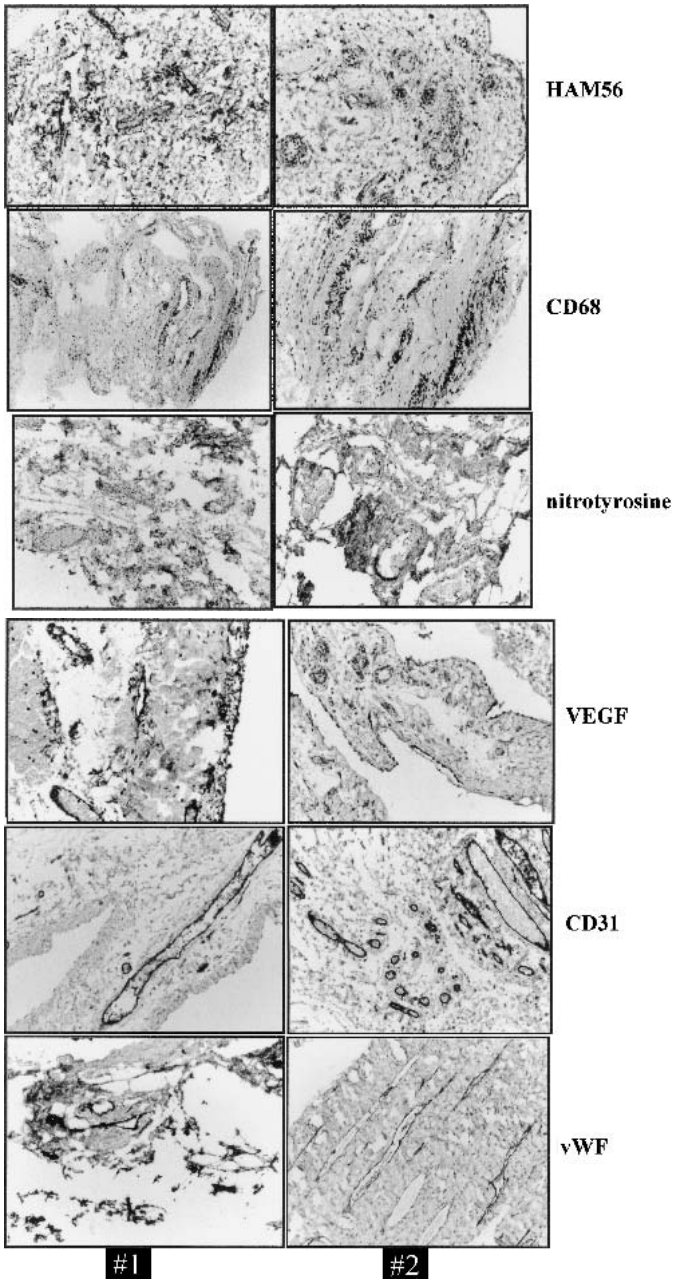

Fig. 2. Human adhesions are dynamic structures containing inflammatory cells, oxidants, angiogenic growth factors, and blood vessels. Formalin-fixed human adhesion tissues were embedded in paraffin and sectioned (4 μm). Sections were immunostained (primary antibody indicated in parentheses) to visualize macrophage (anti-CD68 and HAM56), ROS/oxidant footprint peroxynitrite (anti-nitrotyrosine), angiogenic growth factor VEGF (anti-VEGF), and blood vessels (endothelial cells; CD31 and vWF). Brown stain (diaminobenzidine tetrahydrochloride) represents the antigens of interest. Images shown are from two subjects i.e., #1 & #2. ROS: reactive oxygen species; VEGF: vascular endothelial growth factor; vWF: von Willebrand factor.

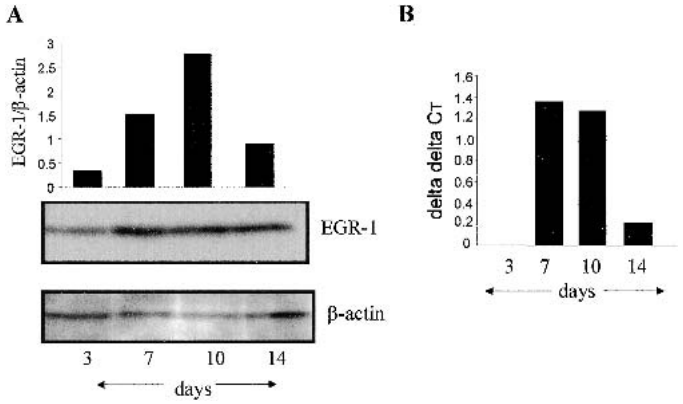

Fig. 3. Activation of ROS/oxidant sensitive early growth response-1 (EGR-1) gene expression in rat peritoneal adhesions. The kinetics of EGR-1 protein and mRNA expression was assessed in rat adhesion tissue as a marker of ROS/oxidant-induced gene expression. **A.** Western blot analysis of EGR-1 protein. The blots were re-probed with β-actin to show comparable loading. **A,** top: For quantification, densitometry was performed. EGR-1 expression is in arbitrary units and data are normalized to respective amount of β-actin protein. **B.** mRNA expression of EGR-1 detected by real-time polymerase chain reaction. For quantification, the delta delta Ct method that normalizes to a housekeeping gene was used.

EGR-1 expression peaks on day seven and gradually declines thereafter.

Discussion

Adhesions are generally viewed as nonfunctional scar tissue [14, 15]. This study presents the first evidence demonstrating ongoing and acute/chronic inflammatory processes, including the presence of large number of macrophage and footprints of macrophage oxidative metabolism, i.e., peroxynitrite. Furthermore, the presence of VEGF, a key angiogenic factor, together with a large number of mature and immature CD31/vWF-positive blood vessels was evident. As opposed to previous views [14, 15], these data support a previous finding that adhesions are dynamic, regenerating structures [16]. To study this dynamic process of de novo tissue formation, we established a novel experimental model of surgical adhesion formation in rats. With that model we demonstrate the kinetics of macrophage infiltration to the adhesion tissue as well as activation of EGR-1, a ROS-sensitive transcription factor [12].

It is generally believed that adhesions are the end result of aberrant peritoneal healing and as such, they may represent fibrous avascular scar tissue [14, 15]. Our study, however, demonstrates that human adhesions are highly cellular and vascularized structures containing inflammatory cells, oxidants, and angiogenic factors such as VEGF. A previous study has extensively characterized the cellular composition, vascularity, and extracellular matrix distribution of human peritoneal adhesions [16]. Consistent with our findings, the study reported that adhesion tissues are highly vascularized, containing well-developed arterioles, venules, and capillaries. They also observed that most of the adhesion tissues studied were also innervated containing nerve fibers, with both myelinated and non-myelinated axons [16]. There appeared to be no correlation between the estimated maturity or site of each adhesion and its histological appearance [16].

We have previously shown that ROS generated by phagocytic cells at the site of tissue injury serve as major signaling molecules regulating expression of VEGF and wound angiogenesis [6, 7]. This finding, together with the observation that adhesion tissue contains the ROS-inducible angiogenic factor VEGF and extensive vascularization, led us to investigate whether the adhesion tissue contains ROS-generating phagocytic cells and footprints of ROS. Presence of macrophage and nitrotyrosine led us to hypothesize that macrophage and macrophage-derived oxidants drive adhesion tissue formation by inducing angiogenic responses.

We established a novel experimental model of peritoneal adhesion in rats. Our objectives were to establish an objective, reproducible model of postoperative intraperitoneal adhesion to study the dynamic process of de novo adhesion tissue formation. In this model, adhesion tissue formation was evident from day 3 onwards. By day 10, a thick band of newly synthesized tissue around the implanted Marlex piece was observed. Laser Doppler analysis of this tissue indicated that the adhesion tissue was well perfused (data not shown). It has been reported that after surgical injury, there is an initial infiltration of fibrin. However, after that initial influx, neutrophils and macrophages are recruited into the site (24–72 hours post-event) [17]. We observed peak infiltration of macrophage in the adhesion tissue between postoperative days 3 and 5 in our experimental system.

EGR-1 is activated by ROS/oxidants partially via a Ras/Raf/ERK signaling pathway [12]. Within the peritoneum, after injury and inflammation, ROS generated by the respiratory burst process [18] may activate intracellular pathways leading to upregulation of EGR-1. This ROS-inducible gene is also implicated in the regulation of angiogenesis and wound healing [19, 20]. Gene therapy using EGR-1 applied to rodent dermal wounds show accelerated wound healing [13]. Given the mechanisms of EGR-1 induction and its ability to promote VEGF transcription, it may be viewed as an important transcription factor in adhesion formation. Further experiments are required to elucidate the specific mechanisms involved in the expression of EGR-1 and whether its expression plays a central role in peritoneal adhesion formation and maintenance.

In summary, the current study provides the first evidence of colocalization of inflammatory cells and their derivative ROS in human peritoneal tissue. The presence of ROS was also evident in tissues generated by an experimental rodent peritoneal adhesion model where activation of the ROS-sensitive transcription factor EGR-1 was observed. Along with ROS, VEGF and a large number of mature and immature CD31/vWF positive blood vessels were present in the adhesion tissue. These observations suggest that adhesions are not nonfunctional scar tissues, but that they are highly cellular, vascularized, and dynamic structures containing inflammatory cells, oxidants, and angiogenic factors. Further understanding of the molecular mechanisms underlying the pathogenesis of peritoneal adhesions will lead to the formulation of potent strategies to subdue adhesion formation. Although approaches to suppress monocyte infiltration to the wound site may impair the healing of surgical wounds [21], antioxidant strategies to negate the sustained function of ROS at the site of peritoneal adhesion formation may prove to be productive.

Résumé. Les adhérences postopératoires sont des complications fréquentes et sévères de la chirurgie abdominale générale et gynécologique qui peuvent provoquer une douleur abdominale chronique, une occlusion intestinale ou être une cause d'infertilité. La physiopathologie spécifique des adhérences reste mystérieuse et l'attitude thérapeutique actuelle est surtout préventive: technique chirurgicale méticuleuse et utilisation des barrières physiques de protection, sous forme de gels ou de solutions. Nous avons rapporté que les espèces réactives d'oxygène (ROS), générées au site de la lésion par les cellules phagocytaires, servent de molécules messagères majeures régulant l'expression de VEGF et par conséquent, la cicatrisation. Nous avons émis l'hypothèse que les adhérences péritonéales sont le résultat d'une sur-cicatrisation et que, tout comme dans la cicatrisation normale des plaies, les ROS sont impliquées dans leur pathogenèse. Nous avons mis en évidence dans le tissu des adhérences les empreintes de ROS et le facteur VEGF angiogénique, ROS inductible. Un modèle expérimental d'adhérences péritonéales a été établi chez les rongeurs pour étudier la dynamique de l'expression génétique induite par les ROS pendant la formation *de novo* des adhérences. L'analyse immunohistochimique a démontré la présence de ROS/oxydant et de macrophages dans le tissu péritonéal humain. On a mis en évidence la présence de ROS et du facteur de transcription EGR-1, ROS-sensitif, dans le modèle expérimental d'adhérences péritonéales du rongeur. Etaient présents dans le tissu d'adhérence, à coté des facteurs ROS, le facteur VEGF et un grand nombre de vaisseaux CD31/vWF-positifs mature et immature. Ces observations vont à l'encontre de l'hypothèse que les adhérences sont constituées de tissus dont la cicatrisation est défectueuse. Le modèle animal utilisé ici pourrait être une approche intéressante pour étudier de façon reproductible et objective les mécanismes moléculaires derrière le processus dynamique de la formation *de novo* d'adhérences.

Resumen. Las adherencias peritoneales postoperatorias son complicaciones comunes y serias de la cirugía abdominal y ginecológica que pueden causar dolor abdominal crónico, obstrucción del intestino delgado e infertilidad. El conocimiento de la fisiopatología específica de las adherencias peritoneales sigue siendo elusivo y el tratamiento actual ha sido relegado hacia la prevención mediante una técnica quirúrgica meticulosa y el uso de barreras físicas protectoras, gels y soluciones. Nuestro grupo ha informado que las especies reactivas de oxígeno (ERO) que generan las células fagocíticas en el lugar de la lesión tisular sirven como moléculas señaladoras mayores de la regulación de la expresión del factor de crecimiento endotelial vascular, (vascular endotelial growth factor, VEGF) y la subsiguiente reparación de la herida. Hemos planteado la hipótesis de que las adherencias peritoneales son el producto de un exceso de cicatrización de las heridas quirúrgicas y que, como en la cicatrización de la herida, los ERO están implicados en su patogénesis. Procedimos a estudiar la presencia de huellas de ERO y del factor angiogénico inducible por ERO, el VEGF, en tejidos adherenciales humanos. Se estableció un modelo experimental murino de adherencias peritoneales a fin de estudiar la expresión genética inducida por ERO en el curso de adherencias formadas *di novo*. El análisis inmunohistoquímico demostró la presencia de oxidante/ERO y macrófagos en el tejido adherencial humano. La presencia de ERO, y del factor EGR-1 de transcripción ERO-sensitivo también apareció evidente en el modelo experimental murino de adherencias peritoneales. ERO, VEGF y un número de vasos sanguíneos maduros e inmaduros positivos para CD31/vWF, se encontraron presentes en el tejido adherencial. Estas observaciones no son consistentes con el planteamiento de que las adherencias son tejido cicatricial no funcional. Este nuevo modelo murino de adherencias puede significar un aproche útil para estudiar en forma reproducible y objetiva los mecanismos moleculares involucrados en el proceso dinámico de formación de tejido adherencial.

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References

1. Liakakos T, Thomakos N, Fine PM, et al. Peritoneal adhesions: etiology, pathophysiology, and clinical significance. Recent advances in prevention and management. Dig. Surg. 2001;18:260–273
2. Menzies D, Ellis H. Intestinal obstruction from adhesions—how big is the problem? Ann. R. Coll. Surg. Engl. 1990;72:60–63
3. Johns DB, Keyport GM, Hoehler F, et al. Reduction of postsurgical adhesions with Intergel adhesion prevention solution: a multicenter study of safety and efficacy after conservative gynecologic surgery. Fertil. Steril. 2001;76:595–604
4. Vrijland WW, Tseng LN, Eijkman HJ, et al. Fewer intraperitoneal adhesions with use of hyaluronic acid-carboxymethylcellulose membrane: a randomized clinical trial. Ann. Surg. 2002;235:193–199
5. Sen CK, Khanna S, Venojarvi M, et al. Copper-induced vascular endothelial growth factor expression and wound healing. Am. J. Physiol. Heart Circ. Physiol. 2002;282:H1821–H1827
6. Sen CK, Khanna S, Gordillo G, et al. Oxygen, oxidants, and antioxidants in wound healing: an emerging paradigm. Ann. N. Y. Acad. Sci. 2002;957:239–249
7. Sen CK, Khanna S, Babior BM, et al. Oxidant-induced vascular endothelial growth factor expression in human keratinocytes and cutaneous wound healing. J. Biol. Chem. 2002;277:33284–33290

8. Roy S, Khanna S, Bickerstaff A, et al. Oxygen sensing by primary cardiac fibroblasts: a key role of p21Waf1/Cip1/Sdi1. Circ. Res. 2003;92: 264–271
9. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T))method. Methods 2001;25:402–408
10. Sen CK, Khanna S, Roy S, et al. Molecular basis of vitamin E action. Tocotrienol potently inhibits glutamate-induced pp60(c-Src) kinase activation and death of HT4 neuronal cells. J. Biol. Chem. 2000;275: 13049–13055
11. Beckman JS, Beckman TW, Chen J, et al. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. Proc. Natl. Acad. Sci. U. S. A. 1990;87: 1620–1624
12. Wung BS, Cheng JJ, Chao YJ, et al. Modulation of Ras/Raf/extracellular signal-regulated kinase pathway by reactive oxygen species is involved in cyclic strain-induced early growth response-1 gene expression in endothelial cells. Circ. Res. 1999;84:804–812
13. Bryant M, Drew GM, Houston P, et al. Tissue repair with a therapeutic transcription factor. Hum. Gene Ther. 2000;11:2143–2158
14. Ellis H. The aetiology of post-operative abdominal adhesions. Br. J. Surg. 1962;50:10–16
15. Jirasek JE, Henzl MR, Uher J. Periovarian peritoneal adhesions in women with endometriosis. J. Reprod. Med. 1998;43:276–280
16. Herrick SE, Mutsaers SE, Ozua P, et al. Human peritoneal adhesions are highly cellular, innervated, and vascularized. J. Pathol. 2000;192: 67–72
17. Ryan GB, Grobety J, Majno G. Postoperative peritoneal adhesions. Am. J. Pathol. 1971;65:117–148
18. Babior BM, Kipnes RS, Curnutte JT. Biological defense mechanisms. The production by leukocytes of superoxide, a potential bactericidal agent. J. Clin. Invest. 1973;52:741–744
19. Fahmy RG, Dass CR, Sun LQ, et al. Transcription factor Egr-1 supports FGF-dependent angiogenesis during neovascularization and tumor growth. Nat. Med. 2003;9:1026–1032
20. Braddock M. The transcription factor Egr-1: a potential drug in wound healing and tissue repair. Ann. Med. 2001;33:313–318
21. Rodgers KE. Nonsteroidal anti-inflammatory drugs (NSAIDs) in the treatment of postsurgical adhesion. Prog. Clin. Biol. Res. 1990;358: 119–129