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Global Gene Expression Analysis of Keloid Fibroblasts in Response to Electron Beam Irradiation Reveals the Involvement of Interleukin-6 Pathway

Mamiko Tosa,*† Mohammad Ghazizadeh,* Hajime Shimizu,* Takashi Hirai,† Hiko Hyakusoku,‡ and Oichi Kawanami*

*Department of Molecular Pathology, Institute of Gerontology, Graduate School of Medicine, Nippon Medical School, Kawasaki, Japan; †Department of Plastic and Reconstructive Surgery, Nippon Medical School Second Hospital, Kawasaki, Japan; ‡Department of Plastic and Reconstructive Surgery, Nippon Medical School, Tokyo, Japan

Keloid is a dermal fibroproliferative lesion of unknown etiology that commonly recurs after surgical excision. Postoperative adjuvant electron beam (EB) irradiation has been successfully used to reduce keloid recurrences. To provide new insights into the molecular mechanism behind the effect of EB irradiation, we used a cDNA microarray screening of more than 5000 genes to assess early changes in gene expression between EB-irradiated and non-irradiated keloid and non-lesional dermal fibroblasts. Primary fibroblast cultures from keloid and associated non-lesional dermis obtained from five patients were exposed to 15 Gy EB irradiation and analyzed after 15 min incubation. Early response to EB irradiation showed that 96 (1.8%) genes were modulated 2-fold or more in keloid fibroblasts. Upregulated genes accounted for 29.2% (28 genes), whereas downregulated genes comprised 70.8% (68 genes), indicating a silencing of many genes in keloid fibroblasts after EB irradiation. Many of the downregulated genes play roles in the enhancement of cell proliferation and extracellular matrix production, whereas several of the upregulated genes involves in the promotion of apoptosis and extracellular matrix (ECM) degradation. Using emerging bioinformatic tools and further corroboration, the interleukin 6 (IL-6) signaling pathway was found to be mainly involved in EB irradiation response. We also showed co-expression of IL-6 and its specific receptor (IL-6Rα) in keloid fibroblasts that points to the existence of an IL-6 autocrine loop in these cells. These results suggested that at the molecular level, EB irradiation might hinder keloid formation by regularizing disturbances in the homeostatic equilibrium between inducer and inhibitor activities in the matrix system most likely through the IL-6 pathway. Our study provides clues for the molecular mechanism(s) behind the beneficial effect of EB irradiation in reducing keloid recurrences and may help develop alternative strategies for the therapy and prophylaxis of this lesion.

Key words: cDNA microarray/electron beam irradiation/gene expression/interleukin 6 (IL-6) pathway/IL-6 receptor/keloid fibroblast/non-lesional dermal fibroblast
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Keloid is a fibroproliferative lesion characterized by excessive collagen deposition. It develops as a result of abnormal wound healing in susceptible individuals. Keloid is defined as a scar within the skin that grows beyond the confines of original wound. Many factors such as skin tension, wound infection, racial difference, and genetic predisposition have been implicated in the etiology of keloid lesion (Shiuh *et al*, 1996).

A number of treatment modalities have been employed to overcome keloid or to relieve its symptoms. In general, surgical excision alone has proved unsatisfactory because of a recurrence rate of 45%–100% (Berman and Bieley, 1996). This has led to the development of various adjuvant therapies including radiotherapy (Berman and Bieley, 1996; Maarouf *et al*, 2002; Ragoowansi *et al*, 2003). In a randomized trial for keloid therapy, a combination of surgery and radiotherapy appeared to be more effective (12.5% relapse) than that of surgery and corticosteroid injections (33% relapse) at 12 mo after treatment (Sclafani *et al*, 1996). Although keloid radiotherapy alone without surgery is an exception, early postoperative electron beam (EB) irradiation has proved to be a well-tolerated and effective method in reducing the recurrence rate down to 12%–28% (Lo *et al*, 1990; Mitsuhashi and Miyashita, 1995; Maarouf *et al*, 2002; Ogawa *et al*, 2003). In general, EB irradiation appears to be preferable to kilovoltage radiotherapy when treating keloids, since with fast electrons, it is possible to achieve the most advantageous dose distribution in tissue with least undesirable effects (Maarouf *et al*, 2002). The molecular mechanisms underlying the effect of EB irradiation on keloid lesions, however, remain elusive and a universal protocol for its application has not yet been established. Tarott (1994) proposed that the mode of action of irradiation in the treatment

Abbreviations: EB, electron beam; ECM, extracellular matrix; KF, keloid fibroblasts; NF, non-lesional dermal fibroblasts

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of connective tissue hypertrophy is based on proliferation inhibition and accelerated fibroblast differentiation.

Global gene expression analyses using cDNA microarrays have enabled the dissection of molecular events in the irradiation response of tumor cells (Woong-Yang *et al*, 2002) and normal tissue (Steven *et al*, 2002). On the other hand, the availability of recently developed bioinformatic programs that automatically search the biological literature to predict pathways of interacting genes has facilitated identification of gene regulatory networks (Yonan *et al*, 2003). In this study, we used a cDNA microarray screening of more than 5000 genes in keloid fibroblasts (KF) and non-lesional dermal fibroblasts (NF) to identify genes involved in the early response to EB irradiation. Early alterations in gene expression levels appear more likely to involve genes participating in signaling pathways that determine the cell destiny. We further applied the emerging bioinformatic tools to explore what putative pathway(s) might be involved in EB irradiation response.

Results

Effect of EB irradiation doses on KF We first examined the response of KF to different doses of EB irradiation. The KF responded to EB irradiation dose as a non-linear curve and the cell death increased compared with the control, reaching a peak at 10 Gy dose and slightly lower at 15 Gy dose (Fig 1*B*). When the dose was increased to over 15 Gy up to 30 Gy, no significant change was observed. Thus, a 15 Gy dose was chosen for the cDNA microarray analyses. In addition, a comparable dose has been effectively used in several clinical and experimental studies to prevent keloid recurrences (Lo *et al*, 1990; Mitsuhashi and Miyashita, 1995; Maarouf *et al*, 2002; Ogawa *et al*, 2003).

Gene expression analysis of KF and NF in response to EB irradiation Comparison of gene expression profiles of fibroblasts from the five keloid lesions showed comparable levels of expression for all genes (none showed more than 1.5-fold variability). This was true for the five NF as well. Thus, the variability among the cases was within an acceptable range. Comparison of gene expression profiles between NF and KF revealed that many genes were modulated as depicted by scatter plots (Fig 1*C*, *left panel*). These modulations were considered to be related to the keloid disease. Early response of KF to EB irradiation showed modulations in a number of genes that were mostly downregulated (Fig 1*C*, *middle panel*). Further comparison of gene expression profiles between EB-irradiated NF and EB-irradiated KF revealed amelioration of most of the modulated genes that were seen in the absence of EB irradiation, indicating a potential role for EB irradiation in regularizing gene expression imbalances in KF (Fig 1*C right panel*, see Fig 1*C*, *left panel*). Early response of KF to EB irradiation resulted in 96 "candidate-modulated genes" out of the total of 5171 genes (1.8%; Table S1). Downregulated genes accounted for 70.8% (68 clones), whereas 29.2% (28 clones) were upregulated. Of the 96 genes, 91 (95%) were known genes, 65 (71.4%) being downregulated and 26 (28.6%) upregulated. The other five genes were EST, of which three were downregulated and two were upregulated. Com-

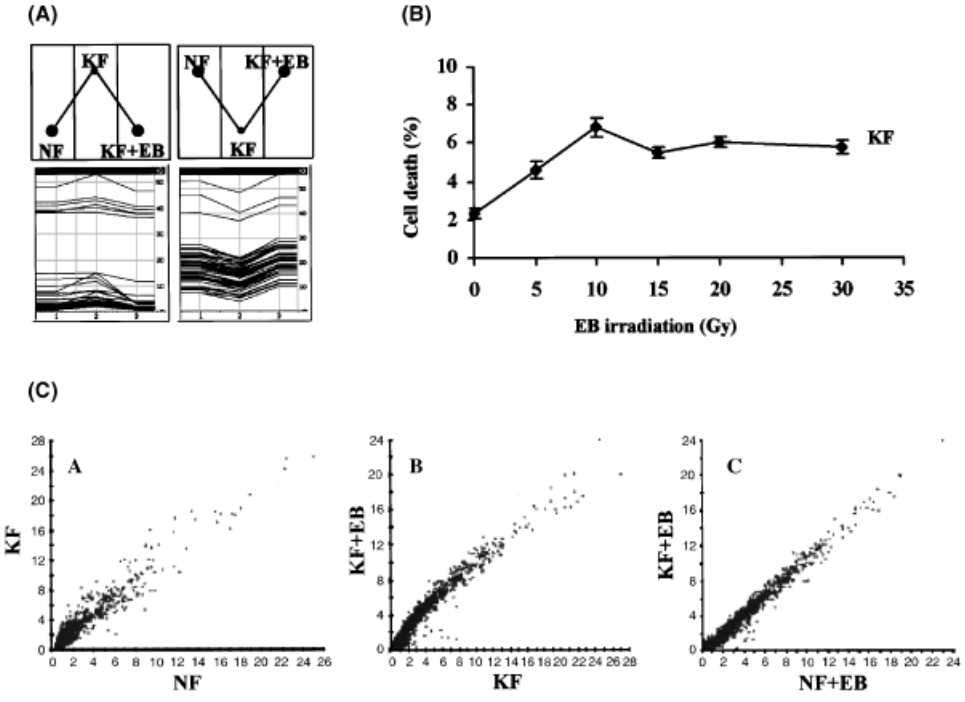


Figure 1
***(A)* Model of retrieving candidate-modulated genes.** Two groups of genes were retrieved using the Vector Xpression 3.0 software: (1) Genes that were upregulated in keloid fibroblasts (KF) compared with non-lesional dermal fibroblasts (NF) and became downregulated in KF after electron beam (EB) irradiation (KF + EB) and (2) genes that were downregulated in KF compared with NF and became upregulated in KF + EB. *Y*-axis denotes the expression intensities. The two groups of genes were then compiled together and considered as the "candidate-modulated genes" in response to EB irradiation. ***(B)* Effect of EB irradiation doses on KF.** KF (1×10^4) were cultured in 25 cm^2 culture flasks using Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum for 24 h. The cells were exposed to single doses of 0, 5, 10, 15, 20, and 30 Gy EB irradiation. After a further 3 d culture in the same medium, the cells were harvested using trypsin/EDTA, washed in PBS, and fixed in 70% ethanol. The cells were stained with propidium iodide (PI), and DNA fluorescence was measured using a flow cytometer. The percentages of cell death were determined and a corresponding curve was constructed. EB irradiation showed a stabilized effect at a more than 10 Gy dose. Data represent mean ± SD for three cases studied. ***(C)* Scatter plots of cDNA microarray analyses.** Left panel: Comparison of gene expression profiles between NF and KF revealed that many genes were modulated. These modulations were attributed to the keloid disease. Middle panel: Early response of KF to EB irradiation showed modulations in a number of genes that were mostly downregulated. Right panel: Further comparison of gene expression profiles between EB-irradiated NF and EB-irradiated KF revealed amelioration of most of the modulated genes that were observed in the absence of EB irradiation, indicating a possible role for EB irradiation in regularizing gene expression imbalances in KF (see *left panel*).

parison of gene expression profiles of NF and KF after EB irradiation did not show specific difference in the response.

Validation of microarray data and verification of downstream targets in interleukin (IL)-6 signal pathway The validity of expression results of some of the genes was confirmed by a semi-quantitative RT-PCR. In general, qualitative agreement between the cDNA microarray and RT-PCR results was quite good, although there were differences in the quantitative extent of the gene expression alterations between the two assay methodologies. This analysis revealed that collagen type I alpha2 (COL1A2), fibronectin 1 (FN1), matrix metalloprotease 2 (MMP2), thymosin beta4 (TMSB4X), protease nexin 1 (PN1), and IL-6 genes were downregulated in EB-irradiated keloid fibroblasts, in agreement with the results obtained by the cDNA microarry analysis (Fig 2*A*). We further documented upregulation of a selected gene of interest, PN1, at mRNA level in the five KF cases as compared with their NF counterparts (Fig 2*B*). In addition, since another gene of interest, IL-6, was downregulated, we sought to examine changes in mRNA expression of two downstream targets in the IL-6 signaling pathway, namely STAT3 and ELK1 and found that these genes were downregulated after EB irradiation (Fig 2*C* and *D*).

Biological function and pathway analysis The biological functions of the "candidate-modulated genes" were diverse and included transcription factors, protein kinases and phosphatases, cell cycle regulators, proteases, cytokines and growth factors, extracellular matrix inducers and inhibitors, apoptotic and anti-apoptotic factors, as well as a number of metabolic genes (Table S1).

To predict what putative pathways might be involved in the response of KF to EB irradiation, a list of "candidate-modulated genes" ($n = 96$) was loaded into the Pathway Assist software. Of those genes, 91 were recognized by the software and were thus subjected to subsequent analysis. These genes consisted of genes related to cytokines and growth factors, extracellular matrix synthesis, proteases and inhibitors, apoptosis, and cell proliferation (Table S1). A subset of functionally interrelated genes was identified and a network of genes graphically represented (Fig 3). This analysis predicted the involvement of IL-6 gene in the response of KF to EB irradiation.

Quantification of IL-6 secretion To determine whether downregulation of IL-6 mRNA in EB-irradiated KF would parallel its protein expression, we measured IL-6 secretion in serum-free cultures of NF as well as non-irradiated and EB-irradiated KF from the five cases by an ELISA method. IL-6 secretion rates were 0.08 ± 0.02 in NF, 0.52 ± 0.04 in non-irradiated KF, and 0.26 ± 0.03 in EB-irradiated KF. IL-6 secretion was found to be significantly increased in KF *versus* NF ($p < 0.01$) or decreased in EB-irradiated KF compared with non-irradiated KF ($p < 0.05$; Fig 4*A*).

Extracellular matrix (ECM) gene expression by addition of IL-6 and EB irradiation or inhibition of IL-6 To confirm that IL-6 signaling pathway is involved in EB irradiation response on ECM gene expression, we examined if IL-6 addition in culture medium abolishes EB irradiation effect on ECM gene expression or alternatively if inhibition of IL-6 could mimic EB irradiation effects on ECM gene expression. We measured mRNA expressions of two principal ECM molecules, COL1A2 and FN1, in KF after treatment with IL-6 peptide at various doses and EB irradiation or anti-IL-6 antibody at various doses. The results showed that addition of IL-6 in culture medium significantly and concentration-dependently interfered with the suppressive effect of EB irradiation on COL1A2 and FN1 mRNA expressions (Fig 4*B*). Furthermore, inhibition of IL-6 by anti-IL-6 antibody resulted

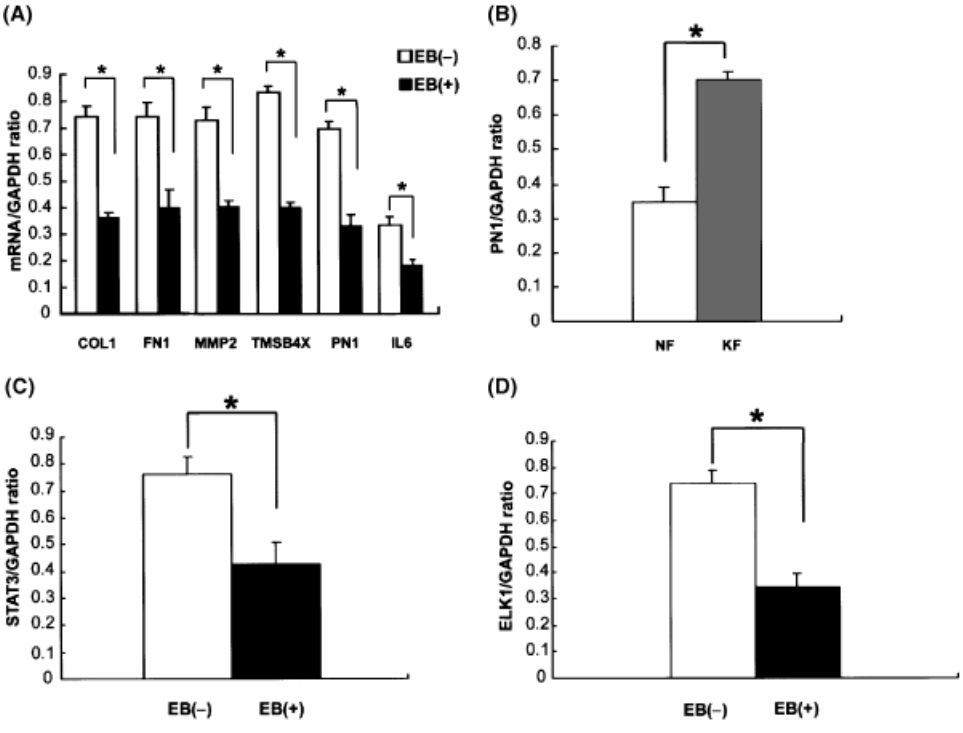


Figure 2
(*A*) Validation of microarray data by semi-quantitative RT-PCR. Intensity values of mRNA expressions from the selected genes normalized against the intensity values of glyceraldehyde 3-phosphate dehydrogenase mRNA confirm the downregulation of each gene after electron beam (EB) irradiation by cDNA microarray analyses. **(*B*) Upregulation of protease nexin 1 (PN1) mRNA in keloid fibroblasts (KF) *versus* nonlesional dermal fibroblasts (NF).** Semi-quantitative RT-PCR showed increased expression of PN1 mRNA in the five cases of KF studied as compared with their NF counterparts ($^*p < 0.05$). **(*C*, *D*) Verification of downstream targets in interleukin (IL)-6 signal pathway.** Measurements of STAT3 (*C*) and ELK1 (*D*) mRNA expression in KF after EB irradiation showed significant decreases.

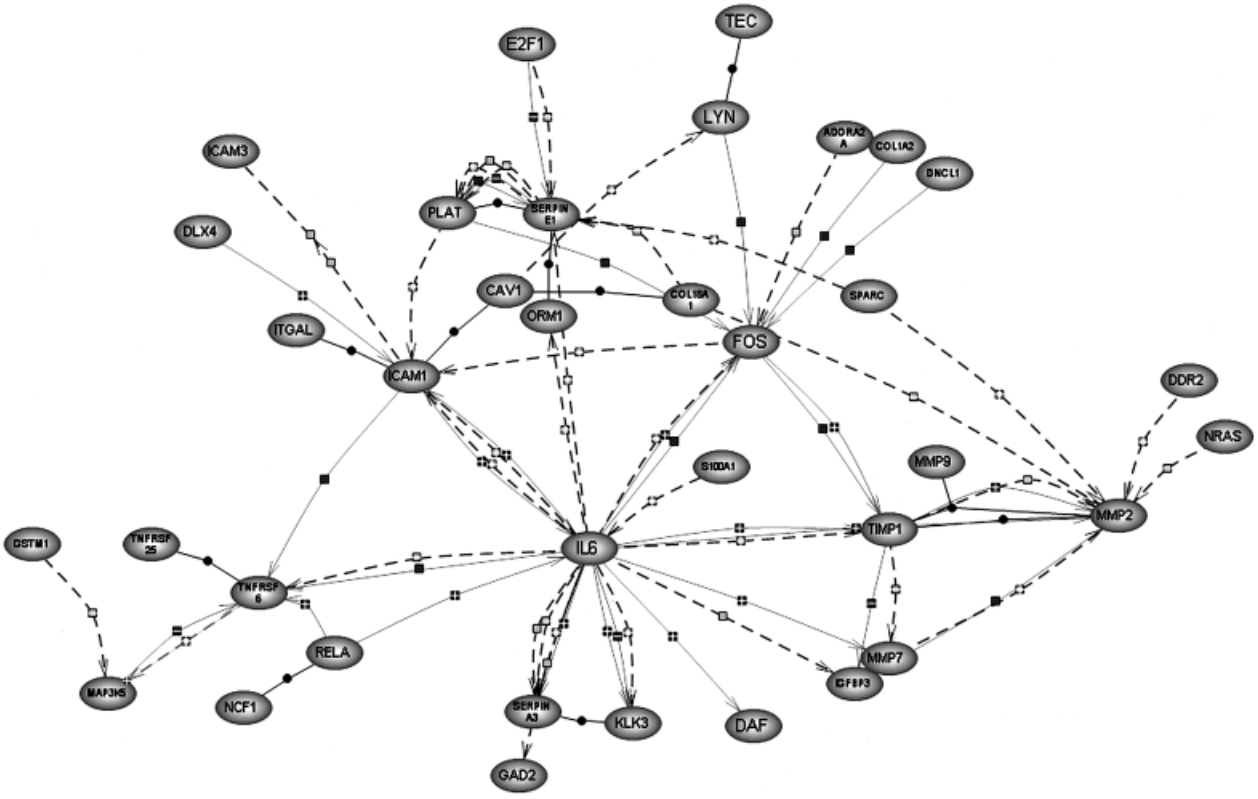


Figure 3
Functional associations among candidate genes modulated in response to electron beam (EB) irradiation in keloid fibroblasts (KF). An original graphical network represented by the Pathway Assist software predicts the involvement of interleukin-6 (IL-6) in response to EB irradiation in KF. Squares marked with "+" or "−" denotes positive or negative effect on the gene in the direction of arrows. The downregulation of IL-6 leads to the downregulation of several genes that promote cell proliferation or extracellular matrix production or inhibit apoptosis in parallel with upregulation of several genes that promote apoptosis or matrix degradation or inhibit cell proliferation.

in concentration-dependent decreases in COL1A2 and FN1 mRNA expressions (Fig 4*C*).

Collagen synthesis on induction or inhibition of IL-6 To provide evidence that IL-6 expression affects increased collagen synthesis, we examined the effects of IL-6 induction or inhibition on collagen type I synthesis using various doses of IL-1β peptide (a known IL-6 inducer) or anti-IL-6 antibody (as inhibitor). We measured procollagen I carboxy-terminal propeptide (PICP) secreted by the KF that reflects the synthesis of type I collagen. Addition of IL-1β peptide or anti-IL-6 antibody in culture medium resulted in a concentration-dependent increase or decrease in the synthesis of collagen type I respectively (Fig 4*D*).

Immunofluorescence and immunoperoxidase stainings for IL-6 and its specific receptor (IL-6Rα) To assess the effect of EB irradiation on the intensity of IL-6 protein expression *in situ*, we compared IL-6 expression between non-irradiated and EB-irradiated KF by visual means using immunofluorescence microscopy (Fig 5*A–E*). A considerably weaker fluorescence intensity of IL-6 expression was observed in EB-irradiated KF (Fig 5*B*) compared with their non-irradiated counterpart (Fig 5*A*). In addition, the intensity of IL-6 expression was greater in KF as compared with NF (not shown).

To reveal whether KF co-expresses IL-6 and IL-6Rα, dual immunofluorescence staining was performed (Fig 5*C–E*). KF demonstrated co-localization of IL-6 and its specific receptor IL-6Rα.

To determine whether the *in vitro* increased IL-6 expression in KF reflected the *in vivo* expression, we assessed IL-6 expression by inmmunohistochemistry in frozen or paraffin sections from 8 additional keloid lesions. KF showed strong expression of IL-6 in 6 (75%), and weak or negative expression each in 1 case (Fig 5*F*). Fibroblasts immediately surrounding the keloid area in the same cases showed no reaction for IL-6. The six cases with strong IL-6 expression also showed moderate to strong expression for IL-6Rα in KF (Fig 5*G*). The other two cases had weak expressions. The immunohistochemical data were thus corresponded to those obtained from cultured cells.

Discussion

Despite extensive research, the nature of keloid lesion remains unclear, yet its mode of therapy bears great clinical importance, because of the frequent recurrences and lack of standard treatment strategy. Cell culture experiments have shown that different populations of fibroblasts may exist in keloid patients and many studies have strongly suggested heterogeneity in cell response of the fibroblasts in the same individuals. In a previous study (Shiuh *et al*, 1996), about 25% of the fibroblasts cultured from keloid

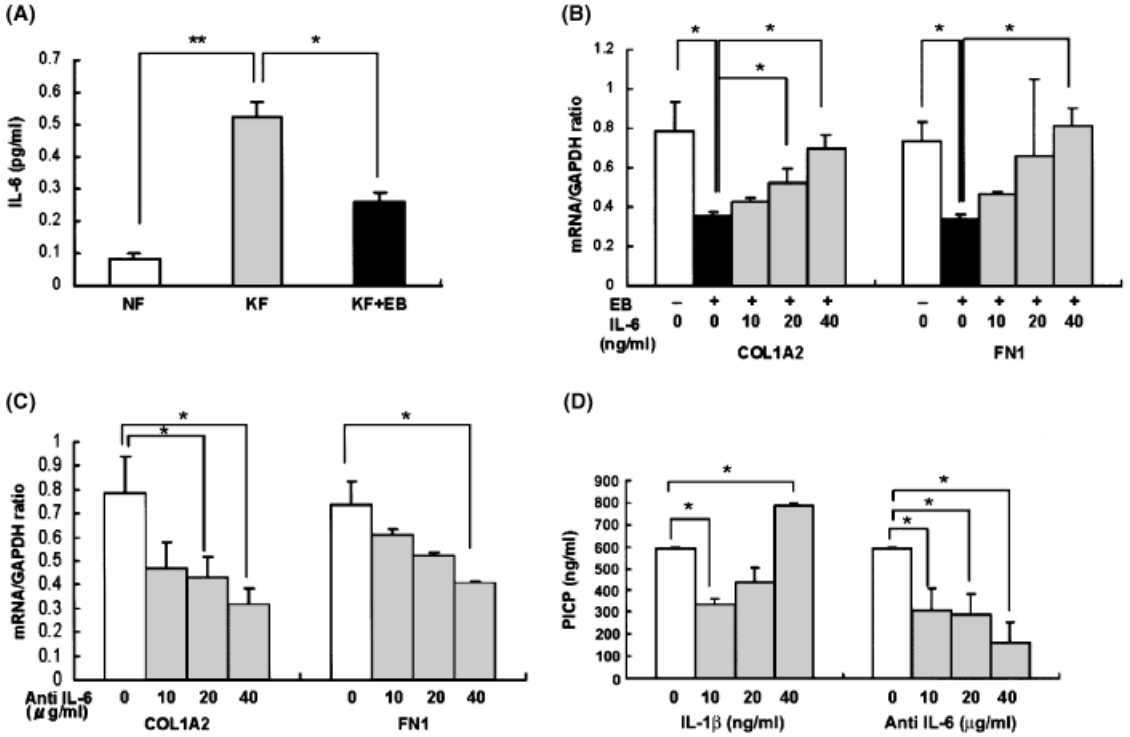


Figure 4
***(A, B)* Electron beam (EB) irradiation effects on interleukin (IL)-6 and extracellular matrix (ECM) components.** Enzyme immunoassay detection of interleukin 6 (IL-6) secretion by non-lesional dermal fibroblasts (NF), keloid fibroblasts (KF), and EB-irradiated KF in the five cases revealed a significantly increased secretion by KF that was decreased after EB irradiation (A). EB irradiation effects on ECM components collagen type I alpha2 (COL1A2) and fibronectin 1 (FN1) mRNA expressions after addition of IL-6 peptide showed a significantly and concentration-dependently increase in the mRNA expressions. Inhibition of IL-6 by anti-IL-6 antibody showed a concentration-dependent decrease in COL1A2 or FN1 mRNA expression (C). ***(C, D)* Effects of IL-6 on ECM components.** Radioimmunoassay detection of procollagen I carboxyterminal propeptides (PICP) in KF after induction (by IL-1β) or inhibition (by anti-IL-6 antibody) of IL-6 revealed a concentration-dependent increase or decease in the synthesis of collagen type I, respectively (* and ** indicate $p<0.05$ and $p<0.01$, respectively).

scar tissues were shown to have increased radiosensitivity compared with those from the normal dermis of the same patients. In contrast, other studies showed that both keloid and normal fibroblasts were sensitive to X-ray irradiation, and there was no significant difference between the two groups (Kyu *et al*, 1994).

Although simple excision and closure result in a recurrence rate of more than 50%, several studies have shown that surgery associated with post-operative EB irradiation provide more than 70% success rate in prevention or recurrence of keloids (Lo *et al*, 1990; Mitsuhashi and Miyashita, 1995; Ogawa *et al*, 2003). The molecular mechanism(s) behind the effect of EB irradiation has, however, not been explored. To provide information on this aspect, we compared gene expression profiles between non-irradiated and EB-irradiated KF considering only those genes that were initially found to be differentially expressed between KF and paired NF from the same patients. Thus, we have analyzed the genes most likely related to keloid disease. Since we were able to compare two tissues from the same individual, we avoided gene expression differences that might arise because of genetic polymorphisms in different individuals. Furthermore, we took advantage of the fact that cultures of fibroblasts from keloid lesion maintain high levels of collagen gene expression for several passages in culture. By these analyses, we could identify several genes that were modulated after EB irradiation (Table S1). Functionally, these modulations included upregulation of several genes that promote apoptosis or matrix degradation or inhibit cell proliferation in parallel with the downregulation of several others that promote cell proliferation or extracellular matrix production or inhibit apoptosis.

Among the modulated genes, downregulation of SERPINE2 or PN1 in KF after EB irradiation attracted our attention. This gene was first discovered and characterized as a serpin-class inhibitor of thrombin released from foreskin fibroblasts (Baker *et al*, 1980). Its main targets are the proteases, including thrombin, urinary plasminogen activator (uPA), and plasmin (Scott *et al*, 1985). It has also activity toward trypsin and urokinase, and binds heparin. Thus, the physiological target of PN1 may include several mediators of matrix metabolism. PN1 binds, possibly by a heparin-like molecule, to the outer surface of cells or to the extracellular matrix and may protect cells and matrix proteins against proteolytic degradation. In our study, downregulation of PN1 in KF after EB irradiation might result in deterioration of matrix metabolism and promotion of matrix degradation. We further documented that PN1 mRNA was aberrantly upregulated in KF compared with NF (Fig 2*B*). In addition, after EB irradiation, other genes including plasminogen activator inhibitor, type 1, member 1 (PAI-1 or SERPINE1), tissue palsminogen activator (PLAT), and urokinase plasminogen activator (PLAU) were also downregulated. In light of the growing evidence that the plasminogen activator and inhibitor system play an essential role in regulating extracellular matrix metabolism in keloid lesions, our results

suggest that this system is most likely to be affected by EB irradiation.

To determine what molecular mechanisms might be involved in the effect of EB irradiation in KF, we sought to apply emerging bioinformatic tools to identify putative gene regulatory interactions between the selected set of differentially expressed genes in KF after EB irradiation. The resulting network of functionally related genes predicted that IL-6, an inflammatory cytokine, may play a pivotal role in modulating response to EB irradiation (Fig 3). Previous studies reported that cytokines could alter fibroblast proliferation, synthesis of extracellular matrix components, as well as other functions that might regulate pathogenesis of autoimmune fibrotic diseases (Elias *et al*, 1990; Ferrarini *et al*, 1990). It was also found that IL-6 produced by fibroblasts may be involved in the pathogenesis of fibrosis associated with rheumatoid arthritis, progressive scleroderma, and pulmonary interstitial fibrosis (Tan *et al*, 1990; Feghali *et al*, 1994; Gurram *et al*, 1994; Shahar *et al*, 1996). In scleroderma and pulmonary fibrosis, overproduction of IL-6 has been linked to the development of fibrosis (Feghali *et al*, 1992; Shahar *et al*, 1996). IL-6 was shown to induce the proliferation of fibroblasts as well (Mihara *et al*, 1995). More recently, increased IL-6 gene expression and overproduction of IL-6 by KF has been reported, suggesting an altered increased autocrine regulation of IL-6 by KF that signifies a central role for this cytokine in the pathogenesis of keloid lesions (Xue *et al*, 2000). In pulmonary fibrosis, contrary to the traditional concept that cytokine secretion by epithelial and inflammatory cells induces parenchymal lung fibroblasts to overproduce collagen, several studies indicate that the pluripotential bone-marrow-derived cells can engraft into lung tissue and produce collagen (Prockop, 1997; Abe *et al*, 2001). Furthermore, a recent study in a mouse model of pulmonary fibrosis provided evidence that most of the collagen-producing fibroblasts in the fibrotic foci in the lung were of bone marrow rather than of local origin (Hashimoto *et al*, 2004). These cells may be recruited to the lung by chemokines secreted from macrophages. Since keloid scar develops after wound healing and several cytokines and chemokines including IL-6 are involved in this process, it is intriguing to speculate that these substances may be responsible for recruiting fibroblast precursors from the bone marrow into the site of wound healing to represent pathologic collagen-producing fibroblasts and overproduce collagen in this disease.

The downregulation of IL-6 gene after EB irradiation in our study appears to have conferred a beneficial effect toward hindering keloid formation. In IL-6 signal transduction pathway (Fig 6), IL-6 binds to its specific receptor IL-6Rα and initiates cellular events, that include activation of JAK kinases and Ras-mediated signaling. Consequently, JAK kinases phosphorylate and activate STAT transcription factors, especially STAT3, that move into the nucleus and activate transcription of genes containing STAT3 response elements. The Ras-mediated signaling through Shc, GRB2 and Sos-1 upstream, and RAF1 and activating MAP kinases downstream activates transcription factors such as ELK-1 and NF-IL-6 (CEBPB). These factors and others such as AP-1 and serum response factor (SRF) that respond to many different signaling pathways regulate a variety of complex promoters and enhancers that respond to IL-6 and other signaling factors. Accordingly, the downregulation of IL-6 by EB irradiation would result in attenuation of these processes,

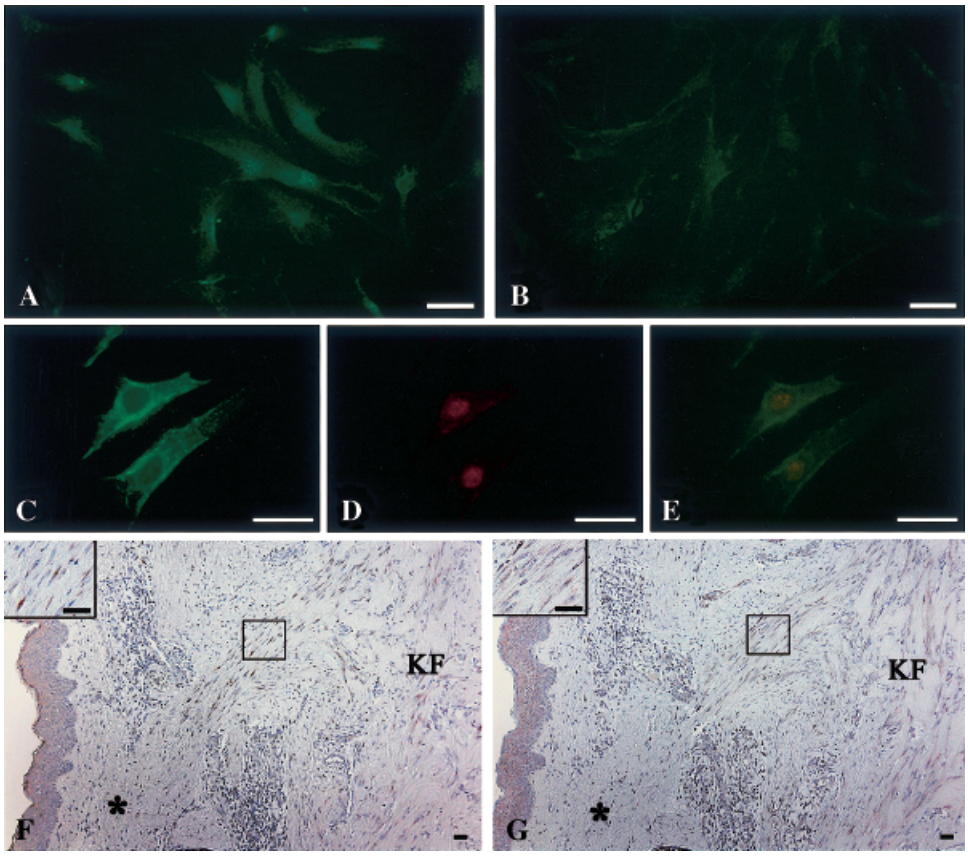


Figure 5
***(A–E)* Immunofluorescence staining for interleukin 6 (IL-6) and its receptor IL-6Rα.** Considerably weaker fluorescence intensity of IL-6 expression was observed in electron beam (EB)-irradiated keloid fibroblasts (KF) (*B*) compared with their non-irradiated counterpart (*A*). In addition, a marked increased intensity of IL-6 expression was found in KF compared with non-lesional dermal fibroblasts (NF) (not shown). Dual immunofluorescence stainings showed that IL-6 (*C*) and its receptor IL-6Rα (*D*) were co-expressed (*E*) in keloid fibroblasts. ***(F, G)* Immunoperoxidase staining for IL-6 and its receptor IL-6R-α.** Immunohistochemical stainings for IL-6 and its receptor (IL-6Rα) in comparable areas from serial parrafin sections of a keloid lesion showed moderate to strong staining for IL-6 (*F*) and its receptor (*G*) in the KF as opposed to negative reaction in fibroblasts immediately surrounding the keloid area (asterisks). Insets: higher magnifications of the squared areas. *Scale bars* = 50 μm.

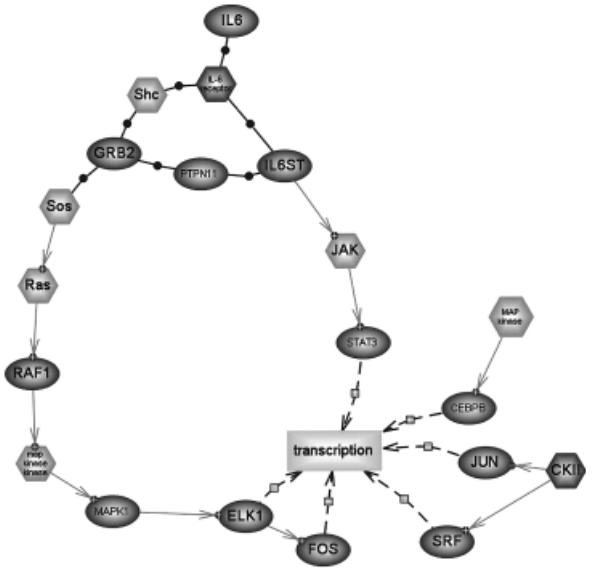


Figure 6
Interleukin 6 (IL-6) signal transduction pathway. Downregulation of IL-6 by electron beam irradiation would result in attenuation of the downstream target genes, leading to the decreased transcription of several genes including those related to the cell proliferation and matrix synthesis.

leading to the decreased transcription of several genes including those related to the cell proliferation and matrix synthesis. Furthermore, we confirmed downregulation of two downstream targets in the IL-6 signal pathway, namely STAT3 and ELK1 in KF after EB irradiation, which adds support to this hypothesis.

In our study, the key elements of the IL-6 signaling pathway, IL-6 and its receptor IL-6Rα were co-expressed in KF that strongly implied an autocrine role for IL-6 in KF. These results were supported by a previous study that also suggested an altered increased autocrine regulation of IL-6 by KF (Xue *et al*, 2000), and further signifies a central role for IL-6 cytokine in the pathogenesis of keloid lesions. In addition, it is likely that the EB irradiation might inhibit increased autocrine regulation of IL-6 by KF. Generally, cytokines other than IL-6 play a role in the regulatory secretion of IL-6 through their interaction with keratinocytes, T cells, and the extracellular matrix. Autocrine regulation of IL-6 is affected by IL-6 itself and other pro-inflammatory factors (Maruyama *et al*, 1995; Boxman *et al*, 1996; Sporri *et al*, 1996). Some studies have shown that interferon (IFN)-γ regulates IL-6 secretion from fibroblasts by stimulating the expression of MHC class II and CD40 (Mourad *et al*, 1992; Yellin *et al*, 1995). IFN-γ has been reported to increase protein production, inhibit DNA synthesis, and increase IL-6 expression in normal dermal fibroblasts (Maruyama *et al*, 1995). On the other hand, ionizing irradiation was found to reduce the IFN-γ mRNA expression (Han *et al*, 2002). Low-dose irradiation, however, is known to exert an anti-inflammatory effect (Roedel *et al*, 2002) and has been shown to induce IFN-γ expression (Shen *et al*, 1991). In fact, low-dose radiotherapy has been effectively used in the treatment of a wide variety of benign conditions. Collectively, the downregulation of IL-6 in response to EB irradiation might be also via the IFN-γ system.

In this study, we used low-passage primary fibroblast cultures to obtain results as close as to the *in vivo* tissue condition. It should be noted that gene expression profiles can be vastly different between the cells in long-term established cultures *in vitro* and whole tissue *in vivo*. Furthermore, the cell-cell, cell-ECM, and cell-host interactions may significantly affect gene expression profile *in vivo* relative to the *in vitro* conditions of established cell lines. These possibilities may also apply when considering if the effects seen with 15 Gy irradiation *in vivo* are reflected when using the same dose in irradiating monolayer cultures of fibroblasts *in vitro*. Fibroblast heterogeneity has been well documented with respect to a variety of functions such as proliferation rate, collagen synthesis, and cytokine production. Phenotypic diversity and differences in morphology and cell size within fibroblast populations also exist. One limitation of our study may be attributed to lack of identification of different subsets of fibroblasts, and variations in the behavior of fibroblasts *in vitro* relative to the *in vivo* environments. In addition, it has been shown that keratinocytes may play an important role in the pathogenesis of keloid (Funayama *et al*, 2003). KF co-cultured with keloid keratinocytes were significantly more proliferative and resistant to apoptosis than those co-cultured with normal keratinocytes, suggesting that keloid keratinocytes may contribute to the keloid formation. In our study, comparison of gene expression profiles of NF and KF after EB irradiation did not show specific difference in the response. Taken together, these findings suggest that the possible benefits of EB irradiation *in vivo* may not be limited to the altered expression of genes in KF cultures and may include changes in keratinocytes and other lesional cell types. Nevertheless, our study identified several putative target genes that can be further investigated in relation to the pathogenesis of keloid lesion.

In conclusion, our differential gene expression and pathway analyses of KF in response to EB irradiation revealed that the inflammatory cytokine IL-6 may play a central role. The involvement of IL-6 was confirmed by the effects shown on ECM gene expression and collagen synthesis in KF through the induction or inhibition of IL-6 expression. We also showed the coexpression of IL-6 and its specific receptor IL-6Rα in KF that points to the existence of an IL-6 autocrine loop in KF. These findings could provide clues for the molecular mechanism(s) behind the beneficial effect of EB irradiation in reducing keloid recurrences and may have implication in other conditions in which fibroblasts produce constitutively increased amounts of IL-6.

Materials and Methods

Tissue specimens Tissue samples from lesional areas of keloid along with associated non-lesional skin borders (2–4 mm peripheral rim) were obtained from five patients with untreated primary keloids during surgery. Informed consents were obtained from all patients. In addition, this study was approved by the Institutional Review Board of the Nippon Medical School and was conducted according to Declaration of Helsinki Principles. All patients were females and ranged in age from 23 to 28 y. All keloid lesions were located on the chest area and had developed after healing of acne vulgaris. There was no sign of acne activity and no patient was under any form of therapy at the time of obtaining tissue samples.

Hematoxylin- and eosin-stained sections from the tissues were histologically examined to confirm keloid and associated non-lesional skin features. There was no evidence of recurrence during a follow up period of 3 y after post-operative EB irradiation.

Cell culture and effect of EB irradiation doses on KF Primary fibroblast cultures were established as previously described (Arakawa *et al*, 1990). Fibroblasts from KFand NF were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum, penicillin (100 IU per mL), streptomycin (100 μg per mL), and amphotericin B (0.25 μg per mL) at 37°C in a humidified 5% CO_2 incubator. The cells were harvested by trypsinization (0.25%) and secondary cultures were maintained in the same medium until reaching 80% confluence. Only low-passage cultures (passage 3–4) were utilized in this study.

To assess the effect of EB irradiation doses on KF, 1×10^4 KF were cultured in 25 cm^2 culture flasks using DMEM supplemented with 5% fetal bovine serum for 24 h. The cells were exposed to single doses of 0, 5, 10, 15, 20, and 30 Gy EB irradiation. After a further 3 d culture, the cells were harvested using trypsin/EDTA, washed in PBS, and fixed in 70% ethanol. The cells were stained with propidium iodide (PI), and DNA fluorescence was measured using a flow cytometer according to a method described previously (Nicoletti *et al*, 1991). The percentages of cell death were determined and a corresponding graph was constructed.

Irradiation and RNA preparation A linear accelerator machine (Mitsubishi Electric Linac EXL-6SP, Tokyo, Japan) capable of generating monoenergicEB at 6 MeV was used. This equipment enables irradiation of the skin to a depth of 0.5–1.5 cm. In addition, the length of the irradiation field is not limited. Since EB has the depth dose characteristic, all deeper structures are spared. Before irradiation, the culture medium was exchanged with fresh medium. The flasks (75 cm^2) containing nearly 80% confluent cells were transferred to the radiology department and each flask received irradiation with a calibrated dose of 15 Gy with a 6.0 MeV EB. The control flasks received only a medium exchange without EB irradiation. After 15 min incubation, fibroblasts from EB-irradiated and non-irradiated flasks were harvested and subjected to RNA isolation using the RNeasy Midi Kit (QIAGEN, Hilden, Germany) based on the manufacturer's instruction. The RNA was treated with DNase and precipitated using 95% ethanol prior to cDNA synthesis. Isolated RNA was electrophoresed through 1.0% agarose–formaldehyde gels to verify the quality of the RNA, and RNA concentrations were determined by UV spectrophotometry.

Microarrays and hybridization The cDNA microarray filters (DermArray, ID 1001) were hybridized according to a protocol developed by the manufacturer (Invitrogen/ResGen, Huntsville, Alabama; http://www.resgen.com). Total RNA (1 μg) was utilized as template for a reverse transcriptase reaction (Superscript II, Invitrogen) to create [^{33}P]dCTP labeled cDNA probes. Reactions were purified by chromatography columns (Bio-Spin 6, Bio-Rad Labs, Hercules, California). The filters were pre-washed in boiling 0.5% SDS for 5 min, placed individually in hybridization roller bottles with 5 mL MicroHyb solution (Invitrogen/ResGen), pre-hybridized with 5 μg denatured poly-dA and Cot-1 DNA (Invitrogen/ResGen) for 2 h at 42°C, and then hybridized overnight with individual [^{33}P]-labeled cDNA probes. The filters were washed for 20 min in hybridization bottles at 50°C each with 2 × SCC for three times, 1% SDS for two times, and 0.5 × SCC/1% SDS for one time. Moist filters were wrapped individually with plastic wrap, carefully oriented and exposed to phosphor-storage screens (BAS cassette 2040, Fujifilm, Tokyo, Japan) for 3 h. Exposed screens were imaged (Molecular Imager FX, Bio-Rad) and tiff files imported into Pathways 4.0 software (Invitrogen/ResGen) for image alignment, translation of the raw hybridization intensities, and comparative analyses. For each gene, a ratio was generated by dividing the normalized expression intensity in EB-irradiated fibroblasts by the normalized intensity of the same gene in non-irradiated counterpart fibroblasts. Genes whose expression changed greater than 2-fold (up or down) in each comparison pair were considered to be differentially expressed. The experiments were conducted independently for two to three times on different filters. For each gene, the ratios for the five cases of KF or NF were averaged.

Analysis of microarrays We first determined the gene expression intensity profiles of: (1) NF, (2) KF, and (3) EB-irradiated KF (KF + EB). After normalization of the data, the expression profile of NF was compared with that of KF and two groups of upregulated and downregulated genes were extracted. These were considered as keloid disease-related genes. Similarly, the expression profile of KF was compared with that of KF + EB and two groups of upregulated and downregulated genes were extracted. These were considered as EB irradiation-related genes. The resultant four groups of modulated genes with greater than 2-fold changes in normalized intensities were exported to Excel files and analyzed using the Vector Xpression 3.0 software (World Fusion, Tokyo, Japan) for comprehensive gene expression analysis. The ratio of each of the upregulated or downregulated keloid disease-related genes was compared to the ratio of the same gene (when present) from the list of EB irradiation related genes and two groups of genes were ultimately retrieved (Fig 1*A*) and recorded as: (1) genes that were upregulated in KF compared with NF and became downregulated in KF + EB and (2) genes that were downregulated in KF compared with NF and became upregulated in KF + EB. The A and B groups of genes were compiled together and considered as the "candidate-modulated genes" in response to EB irradiation. These modulated genes were also examined by cluster analysis, to assign hierarchical grouping to genes whose expression similarly varied (Eisen *et al*, 1998).

Semi-quantitative RT-PCR Some of the cDNA microarray results were verified by a semi-quantitative RT-PCR. Selection of genes was limited to the most frequently represented genes (those with a "present call" in at least four of five keloid cases). RNA samples from the same stocks were used for the cDNA microarray and RT-PCR analyses. From each sample, 1.5 μg of total RNA was reverse-transcribed using the gene-specific primers. The number of PCR cycles was optimized in each case to ensure that product intensity fell within the linear phase of amplification. The primer pair sequences were: COL1A2 forward 5′-ATTAACTCCTTCCCCCGTTC-3′ (nucleotides 149–168), reverse 5′-TGGCAAAGAACAAATGCTGA-3′ (nucleotides 663–644); FN1 forward 5′-CGGAAAAGGAGAATTA-AGTG-3′(nucleotides 5–25), reverse 5′-GCCTTCCAAAGAAACGA-TT G-3′ (nucleotides 332–313); MMP2 forward 5′-TGGG-CAACAAA TATGA GAGCTG-3′ (nucleotides 1356–1377), reverse 5′-CGGC-ATCCAGGTTATCGGG-G-3′ (nucleotides 2128–2147); TMSB4X forward 5′-GCCTTCCAAAGAAACGATTG-3′(nucleotides 64–83), reverse 5′-ATTACAGCCTGCAGGACACC-3′ (nucleotides 565–543); PN1 forward 5′-GGTCCTCGTCAACGCAGTGTA-3′ (nucleotides 777–796), reverse 5′-TGGCAGACAGCG GAGTGG-3′ (nucleotides 1292–1273);IL-6 forward 5′-ACAGCCACTCACCTCT TCAG-3′ (nucleotides 194–213), reverse 5′-CCATCTTTTTCAGCCATCT TT-3′ (nucleotides 341–361). In addition, mRNA expressions of two downstream targets in the IL-6 signal pathway, STAT3 forward 5′-AAGGACATCAGTGGCAAG-3′ (nucleotides 1876–1894), reverse 5′-ACAGGCGGACAGAACA TAG-3′ (nucleotides 2572–2591), and ELK1 forward 5′-GACACGACTCTGCCATCCAT-3′ (nucleotides 2250–2270), reverse 5′-AGGAGGCTTGGGGTCAGAGG-3′ (nucleotides 3092–3112), were examined. The expected PCR products were 515, 328, 792, 500, 516, 168, 715, and 717 bp for COL1A2, FN1, MMP2, TMSB4X, PN1, IL6, STAT3 and ELK1, genes, respectively. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the internal control. The PCR bands were quantitated by densitometry and expressed relative to GAPDH.

Biological function and pathway analysis Gene functions were obtained from the Gene Cards database (http://bioinformatics.weizmann.ac.il/cards/). In addition, we used a recently available

Pathway Assist software (Strategene, La Jolla, California) that allows the user to explore gene interaction networks represented in the ResNet database (a comprehensive database of molecular networks compiled by proprietary natural-language-processing techniques applied to the whole PubMed database) (Yonan *et al*, 2003). We explored functional interrelationships among the genes defined as "candidate-modulated genes" in response to EB irradiation in KF. The structural design of ResNet and Pathway Assist has been described elsewhere (http://www.ariadnegenomics.com). The program allows the user to query the database and construct specific networks relative to genes of interest. The list of "candidate-modulated genes" was loaded into the Pathway Assist. The "Build Pathway" function was used to search for pathways beginning with these genes. The functional relationships were then graphically represented by the software as a network.

ELISA of IL-6 One hundred microliters of a 7×10^4 cells per mL suspension in DMEM with 5% FBS was seeded in 96-well plates in triplicate for 3 d. Then, 100 μL of serum-free medium was added for 12 h. The supernatants were collected for detection of IL-6 using the human IL-6 immunoassay according to the manufacturer's instructions (R&D Systems, Minneapolis, Minnesota). Briefly, the samples were incubated in plates coated with anti-IL-6 antibody for 2 h at room temperature. The plates were then incubated with the antibody against IL-6 conjugated to horseradish peroxidase for 2 h at room temperature. Finally, substrate solution was added to each plate for chromogenic reaction. All plates were read using an ELISA reader set at 450 nm. Each assay was performed in triplicate and the results are presented as means ± SEM.

ECM gene expression by addition of IL-6 and EB irradiation or inhibition of IL-6 KF was seeded on 24-well plates at 7×10^4 cell per well. After reaching subconfluence, the cultures were serum deprived for 24 h. IL-6 peptide (Sigma-Alderich, St Louis, Missouri) was added to a group of cultures at doses 0, 10, 20, and 40 ng per mL of serum-free medium and the cultures were incubated for an additional 12 h and then subjected to 15 Gy EB irradiation. After 15 min incubation, the cells were collected. Anti-IL-6 monoclonal antibody was added to another group of cultures at doses between 0, 10, 20, and 40 μg per mL of serum-free medium and the cultures were incubated for an additional 12 h acnd then the cells were collected. The collected cells from both groups were examined for COL1A2 and FN1 mRNA expressions by RT-PCR. The PCR bands were quantitated by densitometry and expressed relative to GAPDH. The results were presented as mean ± SEM of three independent experiments.

Quantification of PICP on induction or inhibition of IL-6 Collagen type I derives from a larger protein termed procollagen type I, which has propeptide extensions at both ends of the molecule. Before the collagen molecules are assembled into fibers, the propeptides are removed by specific enzymes. The sequence removed from the carboxyterminal, end namely PICP, is secreted by the cells and its measurement reflects the synthesis of type I collagen. KF was seeded on 24-well plates at 7×10^4 cell per well. After reaching subconfluence, the cultures were serum deprived for 24 h. IL-1β (Sigma-Aldrich), a known inducer of IL-6, or anti-IL-6 monoclonal antibody was then added at doses between 10 and 40 ng per mL or 10–40 μg per mL serum-free medium, respectively. The cultures were incubated for an additional 24 h. The medium in each well was collected and processed for PICP radioimmunoassay (RIA) using rabbit anti-human PICP antibody (Orion Diagnostica, Espoo, Finland). The results were expressed as mean ± SEM of three independent experiments.

Immunofluorescence and immunoperoxidase stainings An indirect immunofluorescence method was applied to the primary cultures of KF and their NF counterparts. Cells were seeded in chamber slides (Nalge Nunc International, Naperville, Illinois) at 2.5×10^4 cells per chamber and cultured overnight. Two sets of chamber slides from each sample were prepared, of which one received 15 Gy EB irradiation. After a further 18 h culture, the cells were fixed in cold acetone, washed with PBS, and reacted with 10% normal horse serum for 30 min to inhibit non-specifc binding sites. The cells were incubated overnight with 1:50 dilution of a monoclonal anti-human IL-6 followed by washes in PBS and reaction with 1:100 dilution of a FITC-conjugated horse anti-mouse IgG (Vector Labs, Burlingame, California). Dual immunofluorescence staining was also performed to assess co-localization of IL-6 and IL-6Rα. Here, the cells were incubated overnight with a mixture of 1:50 dilution of a monoclonal anti-human IL-6 and 1:100 dilution of a polyclonal rabbit anti-human IL-6Rα (Santa Cruz Biotechnology, Santa Cruz, California) followed by washes with PBS and incubation with a mixture of 1:100 dilutions of FITC-conjugated horse anti-mouse IgG and Texas-red conjugated goat anti-rabbit IgG (Vector Labs) for 30 min. The reactions were examined using an immunofluorescence microscope.

Immunoperoxidase staining for IL-6 and IL-6Rα was performed on frozen or deparaffinized paraffin sections from 8 additional keloid lesions with location on chest area using a streptavidin–biotin–peroxidase complex method (Niyazi *et al*, 2003). Briefly, endogenous peroxidase in tissues was blocked with 0.3% hydrogen peroxide for 30 min. After washes with PBS, non-specific binding sites in tissues were blocked with 10% normal goat serum and the sections were incubated overnight with 1:50 dilution of monoclonal anti-human IL-6 or ploYclonal rabbit anti-human IL-6R-α antibody. After washes with PBS, biotinylated goat anti-mouse or anti-rabbit IgGs was appplied followed by washing and detection using a streptavidin–biotin–peroxidase staining kit (Histofine, Nichirei, Japan). Diaminobenzidine was used as chromogen. The sections were counterstained with hematoxylin. Negative controls for immunofluorescence and immunoperoxidase methods included ommision of the primary antibody and its substitution with non-immune rabbit serum.

Statistical analysis Statistical analyses were performed using the Student's *t* test for paired data to identify differences within groups before and after a given treatment and Bonferroni's test to identify differences between groups. A p value of less than 0.05 was considered as significant.

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Supplementary Material

The following material is available from http://www.blackwellpublishing.com/products/journals/suppmat/JID/JID23592/JID23592sm.htm
Table S1. Candidate-modulated genes in response to EB radiation in keloid fibroblasts

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Address correspondence to: Mohammad Ghazizadeh, MD, PhD, Department of Molecular Pathology, Institute of Gerontology, Nippon Medical School, Nakahara-ku, Kosugi-cho, Kawasaki 211-8533, Japan. Email: ciem@nms.ac.jp

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