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Aging and Lung Injury Repair: A Role for Bone Marrow Derived Mesenchymal Stem Cells

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ABSTRACT

The incidence of lung fibrosis increases with age. Aging is associated with modifications in the intracellular and extracellular environment including alteration of the extracellular matrix, imbalance of the redox state, accumulation of senescent cells and potential alteration of the recruitment of bone marrow mesenchymal stem cells. The combination of these senescence-related alterations in the lung and in bone marrow progenitor cells might be responsible of the higher susceptibility to lung fibrosis in elderly individuals. The understanding of these age related changes must be considered in the rationale for the development of therapeutic interventions to control lung injury and fibrosis. J. Cell. Biochem. 105: 641–647, 2008.

KEY WORDS: AGING; STEM CELLS; LUNG

The incidence of pulmonary fibrosis increases with age, independent of exposure to common environmental risk factors. Aging is accompanied by multiple systemic alterations including enhanced inflammatory responses, oxidant stress resulting from depletion of anti-oxidants, and the accumulation of senescent cells (Fig. 1). Over the past few years, the recruitment of stem cells has emerged as a possible important repair mechanism that might be affected in chronic fibrotic lung disorders [Forbes et al., 2002]. Consistent with this idea, infusion of a specific stem cell population termed bone marrow derived mesenchymal stem cells (BMDMSCs) attenuates fibrosis and reduces inflammation in a murine model of lung injury [Ortiz et al., 2003; Rojas et al., 2005; Xu et al., 2007b]. In contrast, others have shown that the recruitment of another subpopulation of bone marrow derived cells termed fibrocytes may actually contribute to the development of fibrosis after lung injury [Phillips et al., 2004; Xu et al., 2007a]. Together, these data suggest that bone marrow derived cells might promote repair after lung injury, but only if the type, number, and timing of the recruited stem cells is tightly controlled; the latter being greatly dependent on as yet unidentified host factors. Aging is associated with significant alterations in extracellular matrix composition, elevation of chemokines and inflammatory mediators as well as higher susceptibility to oxidative stress. These changes might contribute to impaired recruitment of bone marrow mesenchymal stem cells or promote the recruitment of bone marrow-derived cells that promote fibrosis instead of repair. All these issues are critical to understand the pathogenesis of fibrosis in order to design therapeutic strategies to prevent or attenuate fibrosis.

LUNG DISEASE AND AGING

There is no compelling explanation for the cause of death in old but otherwise healthy individuals, or any other organism. Dying of natural causes challenges our knowledge of the biological basis of this event. All we can be certain is that the cessation of respiratory and circulatory functions results quickly and irreversible damage to vital organs ending in death.

Even in the absence of disease, there are age related changes in the normal lung. There is a predictable loss of lung volume with advancing age and a decrease in arterial P_{O_2} that is attributed to terminal airway closure and reduced cardiac output. Lung compliance increases with age and the static pressure–volume curve is shifted to the left. Numerous other age-related alterations in physiology could also affect responses to lung injury, including altered baroreceptor activity, reduced adrenergic control of cardiovascular function, and altered autonomic function. An age related failure of normal cardiovascular regulatory mechanisms might be particularly important in responses to sepsis, the most common clinical condition resulting in acute lung injury [Comroe, 1962].

Changes in the composition of the extracellular matrix during aging have been described. In clinical and experimental forms of

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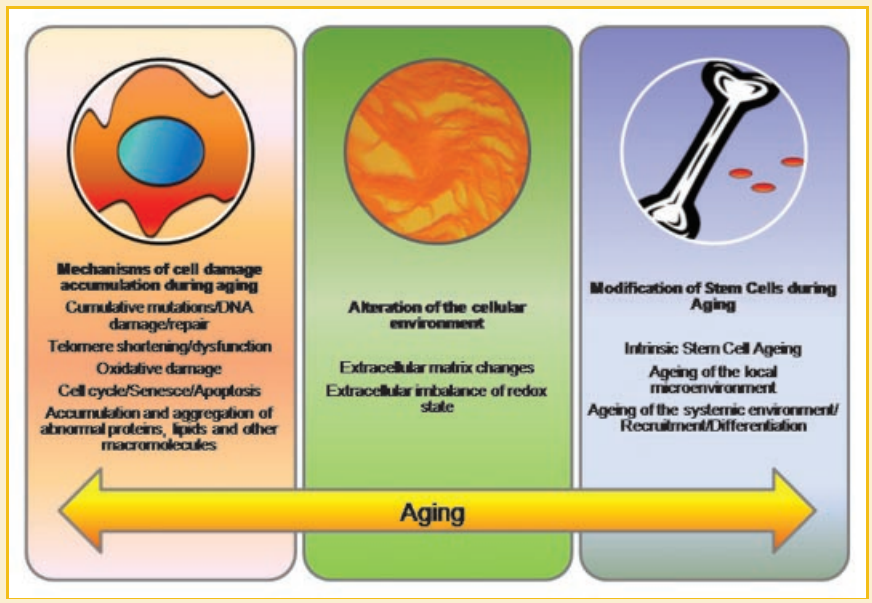

Fig. 1. Mechanisms involved in lung disrepair and fibrosis during aging. Cellular and extracellular modifications during aging may have an impact in the reparative response during lung injury. Age-related alterations involve intrinsic cell changes like senesce and accumulation of cell damage as well as changes in the cellular environment. Senescence of stem cells and changes in the local and systemic microenvironment may also have an impact in the reparative capacity and recruitment of these cell populations during injury.

acute and chronic lung injury, a repair response is triggered that is characterized by, among other things, the elicitation of inflammation, and the increased expression of extracellular matrix components such as fibronectin [Hetzel et al., 2005]. The new matrices are thought to serve as a scaffold for the organization of recruited (i.e., immune cells) and resident lung cells that are activated to exert repair. These matrices also modulate important cell functions related to differentiation, proliferation, and activation [Danen and Yamada, 2001]. In certain cases, however, this response is poorly orchestrated leading to excessive deposition and/or diminished degradation of extracellular matrices and increased fibroproliferation which results in the formation of scar tissue. In pulmonary fibrotic diseases, this scar formation is progressive leading to a relentless course that culminates in loss of lung function and, ultimately, respiratory failure and death. Although many chronic profibrotic lung disorders are caused by environmental and occupational hazards (e.g., asbestosis, allergens, virus infections) or are associated with specific systemic disorders (e.g., connective tissue disorders), others have no identifiable etiologic factor. Idiopathic Pulmonary Fibrosis (IPF) is the prototype of these disorders and most common form of idiopathic interstitial pneumonia with typically poor prognosis and high mortality [Araki et al., 2003]. IPF is typically a chronic disease resulting from aberrant activation of alveolar epithelial cells after injury that provoke pro-fibrotic processes leading to the formation of fibroblastic foci, which in turn overproduce collagen in the extracellular matrix that leads to an irreversible destruction of the lung parenchyma [Selman and Pardo, 2002]. Five-year median survival rates of patients afflicted with IPF are less than 40% and decreases with age. The mean age of diagnosis of IPF is 66 years with an increasing prevalence in individuals older than 75 years [Gee et al., 1990].

In concordance, animal studies document increased susceptibility of the aged lung to fibrotic stimuli. Lung fibrosis induced by cigarette smoke exposure is greater in older mice (8–10 months) than in young mice (2 months) [Matulionis, 1984]. Acquired states that increase tissue turnover are also associated with short telomeres. One study showed that both current and former smokers had shorter telomeres than did age-matched nonsmokers [Valdes et al., 2005]. In addition, there is some evidence that telomeres of the alveolar epithelium in smokers are shorter than those of the alveolar epithelium in nonsmokers [Tsuji et al., 2006].Telomeres shorten with each cell division and ultimately activate a DNA damage response that leads to apoptosis or cell-cycle arrest. Telomere shortening occurs with aging or can be acquired [Harley et al., 1990]. It is therefore possible that somatic telomere shortening, caused by aging or conditions that increase cell turnover (e.g., smoking), could contribute to fibrosis.

The importance of oxidants and oxidant/antioxidant balance in lung injury and fibrogenesis has been studied in animal models of lung injury and IPF patients. However, anti-oxidant therapy remains controversial in the management of fibrotic lung disorders. The redox states of glutathione/glutathione disulfide (GSH/GSSG) and Cys/cystine (Cys/CySS) have been shown to be oxidized with age. Jones [2006] had demonstrate is a cross-sectional study that GSH/GSSG redox as a function of age showed that there is little change in redox state prior to 45 years and then redox becomes oxidized about 0.7 mV/year. In contrast, Cys/CySS redox becomes oxidized after the 25 years of age, more slowly and continuously. Such oxidative changes provide mechanistic switches to control protein conformation, catalytic activity, protein–protein interactions, protein–DNA interactions, and protein trafficking. For instance, oxidative stress can stimulate the expression of fibronectin [Ramirez et al., 2007].

Other signals stimulated by oxidized extracellular E_h Cys/CySS include induction of NF-κB, and Smad3, transcription factors also known for their ability to promote matrix expression. This finding further strengthens the concept that extracellular E_h Cys/CySS can modulate fibroblast phenotype through Smad3/TGF-β1, a known master pathway for tissue remodeling.

Aging is also associated with the accumulation of advanced glycation end products (AGEs) formed by non-enzymatic glycation and oxidation of proteins. AGEs are inducible by oxidative stress and induce oxidative stress. Accumulation of AGEs are found in alveolar macrophages of patients with IPF [Matsuse et al., 1998]. AGE-formation changes the chemical and biological properties of proteins inside and outside of the cell. Binding to specific cell surface receptors induces activation of cellular signaling pathways leading to cellular dysfunction and cell death. Possible pathogenetic factors implicated in the development of pulmonary fibrosis are AGE-modified proteins such as, for example, *N*-(carboxymethyl)-lysine (CML), which is abundantly present in the fibrotic lung tissue [Kasper and Funk, 2001].

Thus, age-related changes in matrix composition, as well as senescence of lung epithelial cells and oxidative stress might contribute to increase the susceptibility of the lung to the development of fibrosis after injury. The mechanisms are still undefined but the lower capacity to repair the epithelium and the promotion of a pro-fibrotic environment will promote scare formation instead of the normal tissue regeneration.

AGING OF MSC

One mechanism of tissue repair recently described in lung and other organs relates to pluripotent stem cells, but their role remains incompletely understood. Bone marrow derived stem cells can be divided into two groups: hematopoietic stem cells (HSC) and mesenchymal stem cells. Bone marrow derived mesenchymal stem cells (BMDMSCs) were first described in the early 1970s by Friedenstein and collaborators [Battino and Ferreiro, 2004], as clonal, plastic adherent cells capable of differentiating into several cell types, such as adipocytes, chondrocytes, and osteoblasts. Recently it has been demonstrated that BMDMSC can also transform into myoblasts, hepatocytes, fibroblasts, lung epithelial cells [Friedenstein and Kuralesova, 1971; Friedenstein et al., 1974; Xu et al., 2007b], endothelial cells, and neural tissue, cells representing all three embryological layers. These cells are also able to support hematopoiesis in culture providing extracellular matrix, cytokines, and growth factors to the HSC.

Characterization of BMDMSCs has been a complicated issue since there are no specific cell surface markers. There are multiple techniques to isolate and expand these cells. Ortiz and collaborators isolated MSCs using immunodepletion for CD34, CD45, and CD11b surface markers [Ortiz et al., 2003]. To isolate our BMDMSC we have used a combination of a method recently described by Peister et al. [2004] using high glucose media that includes fetal bovine serum and horse serum to obtain a group of pluripotential cells expressing Sca-1 antigen and negative for CD45 and CD11b.

Several studies, present evidence that BMDMSCs can migrate and participate in lung repair and assume markers specific for multiple pulmonary cell types [Ortiz et al., 2003; Burnham et al., 2005; Rojas et al., 2005]. In a set of in vitro studies, Spees et al. [2003] demonstrated that by co-culturing mesenchymal stem cells derived from the bone marrow of a GFP (+) mouse with heat shock-injured small airway epithelial cells from a wild type mouse, GFP (+) cells could be made to differentiate into cells with a morphology and molecular phenotype of lung epithelial cells including cells resembling bronchial epithelium. Kotton et al. [2001] examined the effects of intravenous administration of plastic-adherent cultured bone marrow cells from ROSA donors (transgenics expressing β galactosidase) to wild type mice 5 days after intratracheal administration of bleomycin. They detected donor-derived cells in recipient lungs that were morphologically and phenotypically type I pneumocytes. Although these data suggest stem cells are protective, other studies raise the concern that at least a subgroup of adherent bone marrow derived cells (progenitor cells that are CD45+, Collagen+ and CXCR4+ called "fibrocytes") play a crucial role in dysrepair responses that result in persistent fibrosis [Hashimoto et al., 2004; Phillips et al., 2004]. These cells appear to be present in the fibrotic lungs of patients with IPF and in the injured lungs of mice exposed to bleomycin. However, it remains unclear whether the subsets of recruited cells or the conditions to which these cells are recruited or both are important contributors to the pro-fibrotic response.

Several recent studies have documented differences in performance of bone marrow derived stem cells between young and old animals. Administration of endothelial progenitor cells from young, but not from old, mice was reported to restore pathways critical for cardiac angiogenesis in senescent mice without prior bone marrow suppression. In a remarkable study, Conboy et al. [2005] demonstrated that heterochronic parabiotic mice (two mice, one old and one young, surgically joined with shared circulatory systems) restored age related loss of stem cell capacity in blood and liver of the older member of the pair. We have demonstrated the importance of bone marrow derived stem cells in repair of injured lungs in mice [Rojas et al., 2005].

A feature that is important for adult stem cells is the ability to ensure that genetic information is passed on with the highest fidelity to successive generations [Rando, 2006]. The measurement of telomerase length in BMDMSC as a quantification of senescence had not generated conclusive results. Baxter et al. [2004] had reported that, in vitro culture for just 7–10 population doublings (PDs) gives a reduction in telomerase length that, depending on the age of the BMDMSC donor, is equivalent to the loss of more than half their lifespan. They demonstrate that cells derived from an adult donor have already undergone some substantial reduction in their telomere in vivo. Thus, in vitro expansion of BMDMSC may lead to an infusion of cells that have short telomeres and probably severely compromised in their remaining long-term capacity to proliferate and differentiate and home in injured organs. Baxter et al. also have presented a very intriguing data in which two BMDMSC cell lines, after an initial telomere shortening, were able to maintain telomere length for over 40 PDs, suggesting that telomerase is not affected in cell lines only in short term culture

of cells. The potential therapeutic advantages of fetal or very young BMDMSC over adult BMDMSC has been recently demonstrated [Guillot et al., 2007]. First-trimester human fetal BMDMSC represent a developmentally less mature population of stem cells than adult BMDMSC, with a number of characteristics advantageous to the development of cell therapy. These include expression of pluri-potential markers, greater proliferative capacity, and longer telomeres maintained despite higher levels of telomerase activity. All together these results suggest that aging and in vitro culture of BMDMSC are important factors to determine the ability of BMDMSC to initiate the regenerative processes when they are used as a therapeutic approach.

AGING OF EXTRACELLULAR MATRIX

Several studies that analyze changes in the extracellular matrix of the aging lung are focus on collagen and elastin content. These studies show that elastin fiber content is decreased between 2 and 20 months of age, whereas the collagen content is increased in 26-month-old C57BL/6 mice [Huang et al., 2007a,b]. These and related studies suggest that aging is associated with increases in collagen deposition and decreases in elastin in the lung parenchyma; this results in changes in lung compliance [Huang et al., 2007a,b]. The collagen accumulation during natural aging has been associated in rats with reduced proteolytic activity of MMP-1 and MMP-2 with a concomitant increase of tissue inhibitors of MMP (TIMP-1 and TIMP-2) [Calabresi et al., 2007]. Collagen deposition was seen primarily in peribronchial areas with high expression of Collagen-I but not Collagen-III. Interestingly, in the same study the authors showed that basal levels of TGF-β1 mRNA were unaffected by aging.

No changes in fibronectin expression have been reported in lungs of aging rats [Calabresi et al., 2007]. However, studies performed in other organs suggest that fibronectin might be also increased with aging. In the heart, fibronectin is increased in 20-month-old mice when compared to 2- and 12-month-old mice. This change was associated with increased expression of α1 and α5 integrin subunits suggesting that collagen and fibronectin binding integrins were also elevated [Burgess et al., 2001]. The observation that fibronectin is elevated in aging organs is important for the following reasons. Fibronectins are high-molecular weight, multidomain glycoproteins that are assembled into insoluble multimeric matrices by fibroblasts and other cell types. The polymerization of soluble fibronectin into insoluble fibrils within the extracellular matrix is a dynamic, cell-dependent process that is mediated by a series of events involving the actin cytoskeleton and integrin receptors. Several studies indicate that fibronectin modulates collagen gel contraction, and cell migration. In addition, cell–fibronectin interactions may contribute to abnormal tissue remodeling by enhancing and prolonging cell contractility, which can alter the re-epithelization of denuded basement membranes post injury [Hocking, 2002].

Biochemical analysis reveals the existence of different protein isoforms resulting from alternative splicing of a single fibronectin gene. Alternative splicing is developmentally regulated leading to increased expression of ED-A+, ED-B+, and IIICS+ isoforms in fetal tissues and tumor cells. In adult cells, especially in cells induced to terminally differentiate, there is a decrease in fibronectin ED-A+. TGF-β1 has been implicated in the regulation of ED-A+ and ED-B+ splicing since treating fibroblasts with TGF-β1 results in an increase in both ED-A+ and ED-B+ mRNAs [Magnuson et al., 1991]. This is important since TGFβ1 expression is invariably increased in lung injury [Xu et al., 2006]. Some fibronectin isoforms have also been implicated in myofibroblastic transdifferentiation [Thannickal et al., 2003]. It is possible that increases in total fibronectin as well as the relative content of its ED-A+ and ED-B+ isoforms may impact lung repair.

Finally, one potential outcome is that lung recruitment and the function of BMDMSCs during injury may be modulated by their recognition of extracellular matrices via integrins; especially integrins for fibronectin. The susceptibility of aging lungs to the development of fibrosis could be driven by the recruitment of subpopulations of stem cells that promote fibrosis. The recruitment of these cells would be facilitated by aberrant fibronectin-rich matrices that promote the transdifferentiation of these cells into myofibroblasts which, in turn, produce excessive connective tissue matrices resulting in tissue scarring.

AGING AND RECRUITMENT SIGNALS OF IMMUNE AND BONE MARROW CELLS: INFLAMMATION, CYTOKINES AND CHEMOKINES

Clearly documented age-related alterations modify both innate and adaptive immune responses. Aging macrophages have a propensity for increased production of pro-inflammatory mediators [de la Fuente et al., 2004]. In studies in mice, aged animals have an exaggerated lung inflammatory response to inhaled particles and ozone compared to young mice [Sung et al., 2004; Ungvari et al., 2004]. In an interesting study in which bronchoalveolar lavage was performed in young and old healthy human cohorts, older individuals had increased numbers of neutrophils in their lungs indicating low grade inflammation even in clinically healthy older people [Elder et al., 2000].

Immunosenescence (reduction in naive T cells, shrinking T cell repertoire) is a well-recognized phenomenon in man and animals [Meyer et al., 1998]. The well recognized increased susceptibility to respiratory infection and decreased efficacy of vaccination in older people are consequences of the aging immune system [Meyer, 2004]. Especially relevant to the fibrotic response, normal aging has been associated with a shift in T lymphocytes from a predominantly Th1 phenotype to a predominantly Th2 phenotype that is especially evident in frail older persons [Rafi et al., 2003]. Th2 cytokines promote expression of pro-fibrotic factors like TGF-β and Th2 biased animals are more susceptible to lung injury and fibrosis. Humans with chronic fibrotic lung disease also demonstrate a Th2 biased phenotype [Young and Merrill, 1986].

Because the low number of BMDMSC in vivo, studies to determine the function and migration of BMDMSC requires theirs in vitro culture, expansion and adoptive transfer. To analyze in more physiological conditions BMDMSC, we have developed a parabiotic mouse preparation that allows in vivo trafficking of stem cells. In parabiosis, two mice are attached surgically so that they develop a

common circulation (and a chimeric circulating leukocyte pool in both animals). Pair of parabionts where one was transgenic for GFP and the other was not, show approximately 50% of circulating cells expressed GFP in both members of the pair. Parabionts consist of either two young mice (young/young) or one young and one old (24 months) mouse. Bleomycin administration to the non-GFP expressing parabiont result in mobilization and lung recruitment of different bone marrow derived cell populations from the old and young mouse. Further studies will be necessary to determine the potential of these different populations to repair the normal tissue or to induce scar formation.

Fibroblasts are heterogeneous in their phenotype and origin and are key cells during the wound healing process. Fibroblasts can originate from circulating fibrocytes, derived from bone marrow derived cells [Meneghin and Hogaboam, 2007; Strieter et al., 2007]. Fibrocytes express stem and leucocyte cell markers like CD45 but also produce type I collagen. Fibrocytes home and extravasate to sites of tissue injury and differentiate into myofibroblasts [Phillips et al., 2004]. The mobilization and trafficking of fibrocytes as well as leukocyte, and BMDMSC is also regulated by chemokines [Strieter et al., 2007]. Fibrocytes express several chemokine receptors. Human fibrocytes express CCR3, CCR5, CCR7, and CXCR4. Mouse fibrocytes express CCR2, CCR7, and CXCR4. Fibrocytes that express CXCR4 migrate in response to SDF-1 (CXCL12). This chemokine axis has been shown to be important in mediating fibrocyte recruitment in vivo during lung injury and to contribute to fibrosis. Consequently, changes in chemokines expression levels during aging may be important in the pathogenesis of pulmonary fibrosis. The axis CXCL12/CXCR4 has been shown in animal models of lung fibrosis to be important in the recruitment of fibrocytes to the injured lung. For instance, depletion of CXCL12 in the bleomycin-induced pulmonary fibrosis correlates directly with decrease of collagen deposition [Phillips et al., 2004]. Similarly, the use of a CXCR4 antagonist blocked migration of bone derived stem cells in vitro and significantly attenuated lung fibrosis. Studies in IPF patients show that fibrocytes are detected in greater numbers during acute exacerbations. Lungs of IPF patients have increased expression of CXCR4 and SDF-1 compared to normal specimens [Yang et al., 2007]. Aging is associated with a general increase of all chemokine production during injury compared to unstimulated conditions [Pulsatelli et al., 2000]. These studies suggest that high levels of chemokines could be a compensatory mechanism to balance the impaired "specific" immune response and repair during aging. We have studied differences in the frequency of circulating fibrocytes after lung injury in the young and old mice. Our data suggest that circulating fibrocytes are in higher frequency in aging than young animals. In concordance, old mice have persistent upregulation of SDF-1 in the lung after injury. Thus, senescence may be associated with increased ability to mobilize pro-fibrotic bone marrow cells.

SUMMARY

Although the incidence and severity of fibrotic lung disease increase with age, there has been relatively little investigation into the mechanisms of the effects of aging on susceptibility to this disorder. Changes in immune and stem cell function with age are such that they might be expected to increase lung susceptibility to injury and dysrepair. It is well documented that there is a cumulative oxidant burden with age and that oxidant stress is involved in both acute lung injury and fibrotic lung disease. In addition, aging lungs show significant alterations in extracellular matrix composition [Huang et al., 2007b]. These changes might affect stem cell recruitment into injured lungs and promote collagen deposition and fibrosis. One matrix molecule that is increased in fibrotic lung disorders is fibronectin, a matrix glycoprotein implicated in tissue injury and wound healing [Midwood et al., 2006]. Interestingly, fibronectin expression is upregulated in aging tissues [Burgess et al., 2001]. Stem cells seems to adhere, spread, and proliferate more effectively on fibronectin matrices when compared to basement membrane components, and this effect is dependent on the repertoire of integrins expressed on their surface. Furthermore, adhesion to these matrix components leads to alterations in stem cell phenotype with their transdifferentiation into myofibroblasts. Age related changes (Table I), in addition to addressing a fundamental biologic mechanism, can provide a rationale for the development of therapeutic interventions (bone marrow-derived stem cells, chemokine antagonist, anti-oxidants, etc) directed to control lung injury and fibrosis.

TABLE I. Manifestations of Aging During Injury and Repair in the Lung

Localization	Alteration during aging	Potential outcome during repair
Lung epithelial cell	Senescence, telomere shortening	Cell cycle arrest/apoptosis. Decrease in re-epithelization
Fibroblasts	Oxidation	Fibronectin and TGFβ stimulation. Myofibroblast transdifferentiation.
Extracellular matrix	Increase of collagen and fibronectin	Smooth muscle actin expression in fibroblast and bone marrow derived stem cells, decrease in re-epithelization. Potential changes in homing of stem cell populations
	Reduced proteolytic activity of MMPs and increase of TIMPs	Accumulation of collagen
Bone marrow derived mesenchymal stem cells	Senescence?	Altered long-term capacity to proliferate and differentiate
	Altered chemokine recruitment signal	Potential changes in trafficking of stem cell populations
	Modification of integrin repertoire?	Probable changes in homing of stem cell populations
Immune responses	Increased inflammation and chemokine expression	Changes in recruitment of immune cells and bone marrow derived cells
	Bias to Th2 responses	Fibroblast proliferation
	Diminished adaptive immune responses	Higher susceptibility to infection

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