Short report

The effect of chronological age on the inflammatory response of human fibroblasts

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ABSTRACT

The immune system undergoes profound age-related changes, including a gradual increase in the production and circulation of proinflammatory cytokines. Despite the known capacity of fibroblasts to produce cytokines, little is known so far about the inflammatory response of fibroblasts to cellular stress such as viral and/or bacterial infection in the context of aging. Therefore the aim of this study was to analyze the levels of IL6 and IL8 secretion in supernatants of human skin fibroblasts from young and elderly persons. Cytokine and chemokine secretion was analyzed before and after in vitro infection of the cells with Cytomegalovirus (CMV) and/or stimulation with Lipopolysaccharide (LPS). The exposure of fibroblasts to these agents caused inflammatory changes, reflected by the secretion of both the cytokine IL6 and the chemokine IL8 by fibroblasts from young as well as elderly persons. The cytokine/chemokine production induced by either agent alone could be further increased by co-stimulation of the cells with both stimuli. The level of protein secretion was dependent on the chronological age of the fibroblasts. Stimulated human skin fibroblasts from elderly donors produced higher amounts of IL6 as well as IL8 than fibroblasts from young donors. These differences were more pronounced for IL6 than for IL8. The inflammatory response of fibroblasts to stimulation differed among donors and did not correspond to the responsiveness of whole blood derived from the same person.

In summary lifelong CMV-infection may act as an in vivo trigger for inflammatory changes by increasing the inflammatory response to bacterial products such as LPS. It may thus contribute to age-related inflammatory processes, referred to as ‘inflamm-aging’.

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1. Introduction

As individuals age their immune system undergoes profound age-related changes, termed immunosenescence. This is of particular interest as immunological changes with aging contribute to a higher incidence and severity of infectious diseases as well as a decreased efficacy of vaccines in elderly persons (Weinberger et al., 2008). These age-related changes include a gradual increase in the production and circulation of proinflammatory cytokines such as Interleukin-6 (IL6), Interferon gamma (IFNγ) and Tumor necrosis factor alpha (TNFα) with age, leading to a systemic chronic low-grade state of inflammation. This phenomenon is referred to as ‘inflamm-aging’ (Franceschi et al., 2000). Cell types involved in ‘inflamm-aging’ can be innate immune cells, such as monocytes or macrophages, but also highly differentiated, antigen-experienced T cells (Arnold et al., 2011). However, the release of proinflammatory cytokines by non-immune cells such as fibroblasts has also been suggested to play a role in ‘inflamm-aging’ (Mohan et al., 2011). Fibroblasts are ubiquitous, differentiated cells of mesenchymal origin. Due to their capacity to produce cytokines, chemokines (Smith et al., 1997), growth factors (Kahari and Saarialho-Kere, 1997) and other biologically active molecules they are involved in local inflammatory and immune responses. Because of their great importance for the whole organism fibroblasts are an interesting and frequently studied cell type for in vitro investigation (Darby and Hewitson, 2007). In addition to host cells pathogens have been claimed to contribute to ‘inflamm-aging’. The chronic exposure to persistent viruses such as cytomegalovirus (CMV) seems to play an important role (Franceschi, 2007; Vasto et al., 2007). Chronic bacterial infections may also promote inflammation in elderly persons (Gavazzi and Krause, 2002). Despite the known capacity of fibroblasts to produce cytokines, particularly during replicative senescence (Coppe et al., 2008), the effects of aging on the inflammatory response of fibroblasts to cellular stress such as viral and/or bacterial infection have only scarcely been elucidated. In addition it is not known whether some elderly persons have a
After an incubation period of 2 h (37 °C, 5% CO2) the virus suspension was added to con-12-well plate (Techno Plastic Products AG, Trasadingen, Switzerland). Varying virus dilutions were used as controls. Conditioned cell culture supernatants were collected at each time point the culture medium was replaced by the same media. All experiments were performed with human skin fibroblasts of the same passage of cultivation, namely passage 21.

2.4. Cytokine and chemokine profiling

 Supernatants of human skin fibroblasts were analyzed in duplicate for the presence of IL1A, IL1B, IL6, IL8, TNFα, IL10 and IFNγ by commercially available ELISA kits (MabTech, Hamburg, Germany; BioLegend, Fell, Germany or Prometheus, Heidelberg, Germany), each according to the manufacturer’s instructions. Results are presented as ng/mL.

2.5. Western blot analysis

 Cells were washed with cold PBS and whole cell pellets were resuspended in TNE lysis buffer (50 mM Tris HCL pH 8.0, 150 mM NaCl and 1% TritonX100, supplemented with protease inhibitor (1:100, Roche Diagnostics, Basel, Switzerland) and phosphatase inhibitors (1:25, Sigma—Aldrich). After removing unlysed cell components by centrifugation (6000 g, 10 min, 4 °C) protein concentration of lysates was assessed using the Bradford protein assay (Bio-Rad, Vienna, Austria). Samples were mixed with 5× loading buffer (50% glycerol, 5% β-mercaptoethanol, 8.3% SDS, 31.25% 1 M Tris pH 6.8, 0.017% bromophenol blue and 16.67% Aqua dest.), heated for 5 min at 95 °C and loaded onto 4–20% gradient Tris–Glycine precast poly-acrylamide gels (Lonza, Basel, Switzerland). Electrophoresis was performed at constant current (20 mA) until adequate separation of the protein molecular marker (Bio-Rad) was achieved. Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA) using a wet-blot transfer system. Membranes were then incubated with primary Abs against p16 (BD Pharmingen, Franklin Lakes, NJ, USA) and GAPDH (Abcam, Cambridge, UK). Appropriate secondary Abs conjugated with horseradish-peroxidase were used and the immune complexes were visualized using SuperSignal Western Femto substrate (Thermo Scientific, Waltham, MA, USA) and an Alpha Innotech Fluor Chem HD2 chemiluminescence detection unit with AlphaEase FC software (Alpha Innotech). For p16 expression of human fibroblasts densitometrical evaluation of obtained grayscale values normalized to GAPDH was performed. Human foreskin fibroblasts of early and late passages were provided by the Molecular and Cell Biology Division of the Institute for Biomedical Aging Research, Innsbruck, Austria and were used as controls for low and high p16 expression, respectively.

2.6. Statistical analysis

 The arithmetic means± S.E.M. were calculated for each examined group and time point. Comparisons between two groups were analyzed by Mann–Whitney-U-Test using SPSS version 19.0 (SPSS Inc., Chicago, Illinois, USA). Probability values (p) were calculated and a level of p<0.05 was considered as statistically significant. 

3. Results and discussion

3.1. Human skin fibroblasts secrete proinflammatory cytokines and chemokines

 First, conditioned cell culture supernatants of untreated as well as LPS treated human skin fibroblasts from young and elderly persons were investigated after 48 h of cultivation for the presence of IL1A, IL1B, IL6, IL8, TNFα, IL10 and IFNγ. Whereas human skin fibroblasts were able to produce IL6 and IL8, they failed to produce detectable amounts of IFNγ, TNFα, IL10, IL1A and IL1B (data not shown). Based on these results we investigated whether the secretion of IL6 and IL8 depends on the chronological age of the fibroblast donors. Basal IL6 protein secretion was found to be slightly, but not significantly
elevated in the supernatants of fibroblasts from old (0.9 ± 0.3 ng/mL) versus young donors (0.3 ± 0.1 ng/mL), indicating that fibroblasts may contribute to the chronic low-grade inflammatory state found in elderly people, a condition referred to as ‘inflamm-aging’ (Franceschi et al., 2000). Unlike IL6 the spontaneous production of IL8 was found to be slightly, but not significantly lower in human skin fibroblasts of elderly (0.06 ± 0.02 ng/mL) compared to young donors (0.17 ± 0.09 ng/mL).

3.2. Secretion of IL6 and IL8 by human skin fibroblasts after CMV-infection and/or stimulation with LPS is dependent on the chronological age

After our initial studies we wanted to analyze the effect of viral infection and bacterial exposure on the inflammatory responses of human skin fibroblasts. For this reason we infected fibroblasts in vitro with CMV and compared the secretion of IL6 and IL8 to that of...
LPS treated cells after 48 h of treatment (Fig. 1A). A much stronger re-
action to LPS than CMV was observed, but viral infection also induced a
significant release of these inflammatory markers. This is in accordance
with earlier reports which describe that HCMV stimulates the produc-
tion of IL8 and IL6 in human fibroblasts as well as endothelial cells (Browne et al., 2001; Craigien et al., 1997; Grundy et al., 1998;
Zhu et al., 1998). Interestingly, cytokine and chemokine production,
particularly of IL6, could be further increased by co-stimulation of the
cells with LPS and CMV together compared to separate exposure to
each of them, suggesting that latent CMV-infection can increase the
inflammatory response to bacterial products such as LPS (Fig. 1A).

To investigate the genetics of age-related changes in the produc-
tion of IL6 and IL8 by human skin fibroblasts in response to different
extracellular stimuli, human skin fibroblasts from young and elderly per-
sons were infected with CMV and/or stimulated with LPS and cul-
tured for six days. Supernatants were collected every 24 h for six
days and analyzed by ELISA. After in vitro infection with CMV and/
or stimulation with LPS IL6 and IL8 secretion by human skin fibro-
blasts from young and elderly persons increased rapidly within 24 h
and then steadily within the following five days of culture (Fig. 1B).

Human skin fibroblasts from elderly donors produced higher
amounts of IL6 and IL8 than cells from young donors in response to
stimulation. The difference was particularly pronounced for IL6, but
also significant at some time points for IL8 following LPS stimulation.
Increased cytokine production has been described for senescent fi-
broblasts. We therefore analyzed the expression of the senescence
marker p16 in the human skin fibroblasts. Evaluation of p16 protein
expression by Western Blot analysis, as shown in Fig. 2, revealed
low levels of p16 indicating that the skin fibroblasts were not senes-
cent at the time of our experiments.

No difference in p16 expression was observed in skin fibroblasts
from young and elderly donors. Human foreskin fibroblasts (HFF) of
early and late passages were used as controls. Our results are in
agreement with observations by Maier et al. showing that skin fi-
broblasts of elderly donors reach senescence only after at least 50
population doublings, corresponding to approximately 30 passages of
in vitro cultivation (Maier et al., 2007). These results support the
concept that the secretion of IL6 and IL8 by human skin fibroblasts
following infection with CMV and/or stimulation with LPS is affected
by the chronological age of the fibroblast donor. Furthermore these
findings corroborate the hypothesis of an increased proinflammatory
status of aged cells (Coppe et al., 2008; Shelton et al., 1999). Although
the molecular mechanisms responsible for the effect are not yet clear,
the ‘overproduction’ of IL6 and IL8 by fibroblasts of elderly persons
might be the result of in vivo priming with potential epigenetic changes
as a consequence. This possibility has also been previously addressed
for other cell types (Clark and Peterson, 1994). Control experiments
performed with UV-inactivated virus, which can be internalized into
the cells but is not able to replicate, additionally demonstrated that
the induction of IL6 and IL8 requires active viral replication (data not
shown). Lifelong CMV-infection may thus represent an in vivo trigger
for changes leading to an inappropriate production of proinflammatory
molecules, chronic inflammation and an imbalance in the cytokine net-
work between pro- and antiinflammatory cytokines. Increased occur-
cence or progression of age-related diseases may be the consequence.
However, inflammation may not be only detrimental since high
proinflammatory activity of peripheral blood cells of persons older
than 85 years has been linked with a survival benefit (van den
Biggaar et al., 2004). Under which conditions detrimental effects and
under which beneficial ones dominate, remains yet to be elucidated.

3.3. The inflammatory responses to LPS stimulation differs between

For the Leiden 85-plus study whole blood samples of 85-year-old
donors were stimulated with 10 ng/ml LPS for 24 h and LPS induced
secretion of IL6, TNFα and IL1B was determined in ELISA. Inter-
individual differences in the inflammatory cytokine production capacity
were observed and based on these individuals were subdivided into
‘high proinflammatory’ or ‘low proinflammatory’ responders (van den
Biggaar et al., 2004). The capacity to produce high quantities of
proinflammatory cytokines was significantly linked with a survival ben-
efit. In addition IFNγ production of LPS stimulated whole blood was
determined. As skin fibroblasts were available from the same donors,
it was of interest to assess whether a high/low proinflammatory cytokine
production profile of LPS stimulated peripheral blood was accompanied
by a corresponding proinflammatory potential of in vitro LPS stimulated
fibroblasts. IL6 and IL8 secretion by fibroblasts was, however, different
from the inflammatory activity of peripheral blood following LPS stim-
ulation (Fig. 3). Fibroblasts from elderly persons, who were defined as
‘low proinflammatory responders’ – including lower levels of IL6 –
and/or IL8 after 24 h of LPS stimulation as fibroblasts from persons who
were defined as ‘high proinflammatory responders’. The level of IL6 and IL8
secreted by stimulated fibroblasts was not significantly correlated with
IL6, TNFα, IL1B or IFNγ production of whole blood following stimulation
with LPS (Spearman’s rank correlation). These results indicate that the
inflammatory response to LPS varies from cell type to cell type. Whether
this implies that certain organs are more susceptible to the conse-
quences of inflammation in some persons than in others and what
role the genetic background plays versus environmental factors in this
respect is currently subject to further investigations.

**Fig. 2.** p16 protein expression of cultivated human skin fibroblasts of young and elderly persons as well as early and late passage human foreskin fibroblasts. The protein expression of p16 was assessed in cultivated human skin fibroblasts of young and elderly donors by Western Blot analysis. As controls early and late passage (replicative senescent) human foreskin fibroblasts (HFF) were used. (A) The image shows one representative Western blot analysis of p16 expression of early and late HFF and human skin fibroblasts of young and elderly donors. (B) The graph shows the relative expression of p16 compared to late passage (senescent) HFF. Shown are mean values ± S.E.M; n = 3 (early HFF)/n = 5 (young)/n = 8 (elderly). All values were normalized to expression of the housekeeping gene GAPDH (glyceraldehyde-3-phosphate dehydrogenase).
Conflict of interests

The authors declare that none of them has any conflict of interest related to this manuscript.

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