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Correlation between the donor age and the proliferative lifespan of human peritoneal mesothelial cells in vitro: is TGF- β 1 a link?

Running title: TGF- β 1 and human peritoneal mesothelial cell ageing

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Abstract

In the present study we have examined the relationship between calendar age of the donor and the proliferative lifespan and TGF- β 1 production by human peritoneal mesothelial cells (HPMC) in culture. The experiments were performed on primary omentum-derived HPMC isolated from patients undergoing elective abdominal surgery. There was an inverse relationship between the calendar age of the tissue donor and the replicative lifespan of HPMC in vitro ($n=49$, $r= -0.3991$, $p<0.005$). There was also a positive correlation between the donor's age and the magnitude of TGF- β 1 production by first-passage HPMC ($n=28$, $r=0.5400$, $p<0.004$). In turn, the TGF- β 1 levels correlated inversely with the proliferative lifespan of HPMC in vitro ($n=28$, $r= -0.4671$, $p<0.02$). These findings indicate that the reduced proliferative capacity of HPMC isolated from older donors may be associated with increased TGF- β 1 release, which may, in turn, result from the age-related accumulation of senescent HPMC in the peritoneum.

1. Introduction

Transforming growth factor- β 1 (TGF- β 1) is a multifunctional mediator that plays a central role in many biological processes, including cell growth and differentiation, inflammation, angiogenesis, and extracellular matrix accumulation. Because of this pleiotropic activity, the overproduction of TGF- β 1 has been implicated in numerous pathologies, such as atherosclerosis, tumorigenesis and tissue fibrosis (see (Blobe et al., 2000) for review). Interestingly, certain polymorphisms in *TGF- β 1* gene have been found to associate with human longevity (Carrieri et al., 2004). Moreover, increased TGF- β 1 levels have been found both in plasma of elderly people (Forsey et al., 2003) and in fibroblasts undergoing either replicative (Kim et al., 2004) or stress-induced senescence in vitro (Frippiat et al., 2001). It remains unclear, however, whether the changes in TGF- β 1 levels observed during organismal and cellular ageing are in any way related, or if they should rather be considered as two independent phenomena.

Human peritoneal mesothelial cells (HPMC) cover the peritoneal membrane with a fine monolayer that serves many functions. Its anti-wear, lubricating and fibrinolytic properties protect the peritoneum against injury and adhesions. Mesothelial cell-derived mediators control the course of peritoneal inflammation and wound healing. In addition, the mesothelium contributes to the transperitoneal movement of fluid and particulate matter (see (Mutsaers, 2004; Yung et al., 2006) for recent reviews). Vast knowledge of HPMC pathobiology has been largely derived from studies with cultured cells. However, the issue of HPMC senescence was addressed only sporadically (Thomas et al., 1997). We have recently analyzed HPMC senescence in a more systematic manner and found a borderline negative correlation between the proliferative lifespan of HPMC in vitro and the calendar age of individuals from whom the cells were isolated (Ksiazek et al., 2006). We have also demonstrated that senescence of HPMC in vitro is associated with increased TGF- β 1 release

(Ksiazek et al., 2007). In the present study, we have increased the number of tissue donors analyzed to establish more firmly whether there exists a correlation between (i) the calendar age of the donor and the replicative lifespan of HPMC in vitro, (ii) the donors' age and the ability of their HPMC to release TGF- β 1 in vitro and (iii) the production of TGF- β 1 and the replicative lifespan of HPMC in vitro.

2. Materials and methods.

2.1. Cell culture

Human peritoneal mesothelial cells (HPMC) were isolated from the pieces of omentum as detailed by Stylianou et al. (Stylianou et al., 1990). Specimens of omentum were obtained from 49 patients undergoing elective abdominal surgery. Age of the donors ranged from 17 to 77 years. They were not diabetic and had no evidence of peritoneal inflammation and malignancy. The study was approved by the institutional ethics committee and all patients gave their informed consent. Cells were propagated in Earl's-buffered M199 medium (Sigma, St. Louis, MO, USA) supplemented with 10% v/v fetal calf serum (Invitrogen, Karlsruhe, Germany). HPMC were serially passaged until their growth ceased and cells failed to divide within four weeks. The number of cumulative population doublings (CPD) was calculated as described previously (Ksiazek et al., 2006).

2.2. Measurement of transforming growth factor β 1 (TGF- β 1)

TGF- β 1 concentrations in cell culture supernatants were measured with a specific enzyme-linked immunosorbent assay (ELISA), which employed an extracellular domain of human TGF- β receptor type II fused to the Fc region of human IgG (R&D Systems, Abingdon, UK) as a capture. The multi-well modules (Nunc, Roskilde, Denmark) were coated with this recombinant chimeric protein (100 ng/ml in PBS overnight) and then blocked with 10% Roti®-Block (Carl Roth, Karlsruhe, Germany). The samples and standards of human

recombinant TGF- β 1 (ranging from 31.3 to 2000 pg/ml; R&D Systems) were added in duplicates and incubated at 4°C overnight. Captured molecules were detected after the sequential incubation with biotinylated chicken anti-TGF- β 1 (50 ng/ml for 2 hours) and streptavidin-horseradish peroxidase solution (both from R&D Systems). The color was developed following the exposure to the substrate solution of tetramethylbenzidine and H₂O₂ (ICN Biomedicals, Irvine, CA, USA). The reaction was stopped with 2 N H₂SO₄ and the optical density was measured at 450 nm with a reference wavelength of 540 nm. Sensitivity of the assay was calculated to be 15 pg/ml. For the assay the samples were activated by a 15-minute incubation with 1 N HCl and subsequently neutralized with 1.2 NaOH / 0.5 M HEPES.

2.3. Statistics

Statistical analysis was performed using GraphPad Prism™ 4.00 (GraphPad Software, San Diego, USA). Correlations were analyzed using the Spearman test. The comparison of TGF- β 1 release by HPMC from donors below and above 30 years of age was performed with the Mann-Whitney test for unpaired data, and the results were expressed as medians and ranges. Statistical significance was acknowledged when the P-value was less than 0.05.

3. Results

In a group of 49 tissue donors, there was a negative correlation between their calendar age and the replicative lifespan of HPMC in culture (Fig 1A). From this cohort, a subgroup of 28 donors aged 17 to 63 was selected for further analysis. The selection was based solely on the availability of cell culture supernatants in quantities allowing reliable measurement of TGF- β 1. In the subgroup selected, the relationship between the donors' age and the lifespan of HPMC in culture was equally evident (Fig. 1B). Cells from younger individuals (<30 years) reached as many as 7.2 (range 5.1 to 9.6) CPD, compared to 5.6 (range 2.4 to 8.9) CPD

reached on average by HPMC from donors above 30 years of age ($p=0.0597$). Furthermore, we have observed a strong positive correlation between the calendar age of HPMC donors and the production of TGF- β 1 by these cells at their first passage (Fig. 2). Median values for TGF- β 1 release for donors below and above 30 years were 25 pg/ 10^5 cells (range 14 to 99) and 60 pg/ 10^5 cells (range 20 to 116), respectively ($p<0.03$). In addition, we have observed that the magnitude of TGF- β 1 release by first-passage HPMC correlated with the subsequent number of population doublings achieved by HPMC before entering senescence (Fig. 3).

4. Discussion.

It is not entirely clear how the number of cell divisions in vitro relates to the calendar age of the donor. Examining fibroblasts, Hayflick was the first to propose that these parameters were inversely correlated (Hayflick, 1965). Since then several studies have shown such a relationship in many cell types, including keratinocytes (Rheinwald and Green, 1975), endothelial cells (Hoshi and McKeethan, 1986) and adrenocortical cells (Yang et al., 2001). On the other hand, however, the concept has been met with some criticism when the study, in which health status of the donors and biopsy conditions were carefully controlled, failed to demonstrate the correlation between replicative lifespan of skin fibroblasts and the donors' age (Cristofalo et al., 1998). We have previously characterized replicative senescence of HPMC in vitro and on this occasion we have observed a borderline negative correlation between HPMC replicative lifespan and the donor's age (Ksiazek et al., 2006). Since the effect was not entirely certain, we have analyzed more cell preparations to establish more firmly whether or not this relationship exists. Indeed, by increasing the number of subjects examined we have been able to demonstrate clearly the inverse correlation between the lifespan of HPMC and the donors' age. The relationship observed was relatively strong given that biopsy conditions could be controlled only partially. The impact of systemic disease such

as diabetes and uremia was excluded, as well as of overt peritonitis and peritoneal carcinomatosis. Obviously, however, the tissue was sampled from patients with abdominal diseases that frequently – like in intraperitoneal adhesions – involved the omentum.

Furthermore, the mesothelial cell monolayer is an extremely fragile structure, the integrity of which may easily be jeopardized by routine surgical procedures. Prolonged exposure to air, swabbing, pressure from surgical instruments and sutures, lavages with saline - are all the factors with potentially detrimental impact on HPMC (Ryan et al., 1973; von Ruhland et al., 2003; Polubinska et al., 2006).

We have recently shown that the senescent phenotype in HPMC can be induced by TGF- β 1 (Ksiazek et al., 2007). We have therefore analyzed TGF- β 1 release by HPMC in relation to the age of HPMC donor. We found a strong positive correlation in this regard, with early-passage HPMC from older donors secreting more TGF- β 1 than cells from younger individuals. Moreover, these TGF- β 1 levels correlated inversely with the number of HPMC population doublings. One may envisage a scenario in which the percentage of senescent HPMC in the omentum increases with age. Once isolated, these cells produce more TGF- β 1, which in turn inhibits HPMC growth.

The consequences of peritoneal accumulation of aged cells secreting more TGF- β 1 remain hypothetical. While it is now clear that TGF- β 1 plays a pivotal role in wound healing, excessive or prolonged TGF- β 1 expression may lead to increased matrix accumulation and intraperitoneal adhesions and/or fibrosis. In this respect, increased TGF- β 1 expression was detected in post-operative peritoneal adhesions (Holmdahl et al., 2001). Furthermore, by inhibiting HPMC proliferation, TGF- β 1 may contribute to impaired wound healing seen in the aged.

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Figure legends.

Fig. 1. Correlation between the calendar age of the omentum donors and the cumulative number of population doublings achieved by HPMC in vitro. **(A):** results from 49 patients, **(B):** results from a subgroup of 28 patients, HPMC from whom were further analyzed for TGF- β 1 release (see text for details).

Fig. 2. Correlation between the calendar age of the omentum donors and the production of TGF- β 1 by early-passage HPMC.

Fig. 3. Correlation between the basal production of TGF- β 1 by HPMC at passage early and the cumulative number of population doublings.