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Age-related changes in angiogenesis in human dermis

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ABSTRACT

Present research is aimed to examine the number of dermal blood vessels, vascular endothelial growth factor (VEGF), delta-like ligand 4(Dll4) and Jagged-1 (Jag-1) in dermal blood vessels of human from 20 weeks of pregnancy to 85 years old. Numbers and proliferative activity of dermal fibroblast-like cells were also examined. Blood vessels were viewed with immunohistochemical staining for von Willebrand factor or CD31. VEGF, Dll4, Jag-1, and proliferating cell nuclear antigen (PCNA) were detected immunohistochemically. Results showed that the numbers of fibroblast-like cells, PCNA positive fibroblast-like cells, von Willebrand factor positive or CD31 positive blood vessels in dermis are dramatically decreased with age. The intensity of immunohistochemical staining for VEGF or Jag-1 in blood vessels of dermis is increased from antenatal to deep old period. The degree of immunohistochemical staining of dermal blood vessels for Dll4 has gone up from 20–40 weeks of pregnancy to early life period (0–20 years), and further decreased below antenatal values. Age-related decrease in the number of dermal blood vessels is suggested to be due to an impairment of VEGF signaling and to be mediated by Dll4 and Jag-1. It may be supposed that diminishing in blood supply of dermis occurring with age is a cause of a decrease in the number and proliferative pool of dermal fibroblasts.

1. Introduction

Skin of humans gains clinical signs of aging during life. And skin aging is really a great problem for each man. Another connected with this problem is how to prevent or cure skin with appearances of aging. It is clear that we have to know what processes have occurred in skin during aging to dissolve both problems.

Numerous age-associated changes in skin have been discovered. It was found that extracellular matter of dermis obtains deep changes during aging. Collagen and elastic fibers became fragmented and lost their initial organization. Composition and molecular mass of proteoglycans in dermis are also changed during life. Proteoglycans lose their initial molecular mass, and in this condition they can associate less volume of water (Makrantonaki and Zouboulis, 2007; Oh et al., 2011). As a result, skin looks wrinkled, sear and "old". By other words, changes in extracellular matter are underlaid clinical appearances of aged skin. It is evident that changes in extracellular matter of dermis are consequences of processes in other dermal components which regulate synthesis, degradation and trophism of fibers and non-fiber part of the extracellular matter (Makrantonaki and Zouboulis, 2007; Oh et al., 2011).

One of dermal components which regulates metabolism of extracellular matter is dermal fibroblasts. These cells synthesize all components of extracellular matter and provide its degradation. It has been shown that numbers and functional activity of dermal fibroblasts are decreased during aging (Montagna and Carlisle, 1990; Gunin et al., 2011). A decrease in the numbers of dermal fibroblasts is partially due to a slowdown of their proliferative activity (Gunin et al., 2011). Environmental conditions, including sun light, changes in temperature and humidity of air, ecological problems; and metabolic disorders and toxins also play a role in age-induced impairment in dermal fibroblasts condition (Sveikata et al., 2011). However, many factors and mechanisms which lead to a decrease in the numbers and functional activity of dermal fibroblasts still wait their discovering.

Important part which provides metabolism of a tissue is blood supply. Blood brings to a tissue oxygen, trophic and other compounds and takes away products of vital activity. Skin blood supply was investigated and some results were obtained. It was shown that skin of 60–85 years old men has significant decreases in CD31 positive vessel size compared to that of 30–40 years old men, although no major difference was found in the vascular density between these groups (Chung et al., 2002; Kajiya et al., 2011). In skin biopsies obtained from 33 to 82 years old men, it was shown that density of capillaries in papillary layer of the dermis is not changed before 60 years old, but from this age that begins to be

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decreased (Vybohova et al., 2012). In skin of fetuses (13–22 weeks of pregnancy) and in men (34–45 years), the intensity of immunohistochemical staining for CD31 has similar pattern (Coolen et al., 2010). Acute or chronic ultraviolet irradiation which produces experimental skin aging was documented to cause angiogenesis in dermis (Chung and Eun, 2007; Yano et al., 2002). However, afterwards vessels are regressed by apoptosis of endothelial cells and comparable to those without ultraviolet irradiation (Yano et al., 2004). In sun exposed areas of the skin, the density of blood vessels is diminished as compared to sun protected regions (Chung and Eun, 2007; Kajiya et al., 2011). Thus, it is seen that available data concerning blood supply in dermis during age are sometimes contradictory. There are no data on blood vessel numbers in dermis in all age-periods from development to deep old age in the literature. Therefore, one goal of present research is to examine the number of dermal blood vessels from fetal to deep old period of life.

Some mechanisms of age-related changes in blood supply were investigated in the design of ultraviolet-induced skin aging. Ultraviolet irradiation was shown to induce transitory upregulation several angiogenic factors, including vascular endothelial growth factor (VEGF), basic fibroblast growth factor, and interleukin-8 (Bielenberg et al., 1998; Kramer et al., 1993; Sawane and Kajiya, 2012; Strickland et al., 1997), whereas a decreased expression of cytokines with antiangiogenic activity, such as interferon-β and trombospondin-1, has been reported (Bielenberg et al., 1998; Yano et al., 2004). Overexpression of VEGF in the epidermis of transgenic mice resulted in enhanced skin vascularization with increased numbers of tortuous and hyperpermeable blood vessels (Detmar et al., 1998). It was therefore proposed that VEGF released by keratinocytes is a major skin angiogenic factor which regulates ultraviolet-induced angiogenesis in skin (Detmar et al., 1995; Sawane and Kajiya, 2012). However, no evidence was presented that VEGF produced by keratinocytes diffuses through basal lamina of the epidermis to the dermis and changes dermal angiogenesis. Moreover, there is no research which demonstrated VEGF production in dermis during activated or depressed angiogenesis. Therefore, present work was also aimed to examine VEGF in dermal blood vessels from fetal period through life.

Effects of VEGF on angiogenesis are known to be mediated via Notch pathway (Kume, 2012). Notch pathway consists of four types of Notch proteins which are receptors for five ligands, such as Jagged (Jag)-1, Jag-2, Delta-like ligand (Dll)1, Dll3 and Dll4 (Kume, 2012). VEGF induces the expression of Dll4 and leads to the formation of tip cells (Thomas et al., 2013). Tip cells are placed at the end of newly forming blood vessels. Then, Dll4 produced within the tip cells acts on Notch 1 and Notch 4 in adjacent blood vessel cells in which the number of VEGF receptors 2 is decreased, and these cells are formed in stalk cells. Stalk cells are located behind the tip cells and form the trunk of the new vessel. Jag-l in stalk (endothelial) cells activates Notch 3 in neighboring mural cells which are further maturated in pericytes or smooth muscle cells (Thomas et al., 2013). Thus, Dll4 and Jag-1 are the major players in Notch pathway and in new vessel formation. However, there are no data on Dll4 and Jag-1 in human dermis in connection to aging-related changes in blood supply. Therefore, other aim of this work was to examine Dll4 and Jag-1 in human dermal blood vessels from the development to deep old period of life.

2. Materials and methods

All procedures were performed in accordance with the Chuvash State University Ethical Committee Rules for work with human samples.

Skin specimens from frontal surface of the lower part of the neck (from upper corner of standard autopsy skin incision) from human fetuses died antenatally from 20 to 40 weeks of pregnancy, humans who died from different causes from 1 day to 85 years of life were obtained at autopsy. Specimens were fixed in 4% paraformaldehyde and then embedded in paraffin. Transverse 5–7 μm thick sections were prepared.

To count fibroblast-like cells, slides stained with hematoxylin and eosin were used. Slides were initially photographed at 40× magnification of microscopic objective with an Olympus light microscope, an Olympus C3040-ADU camera adapter and an Olympus Camedia 4040z digital camera. Then, pictures were loaded in Sigma Scan Pro 5.0 software (SPSS Inc., Chicago, IL, USA), and areas of tissue for cell counting were selected and then measured (Gunin et al., 2004, 2005). Fibroblast-like cells which have elongated or spindle shaped nuclei with low volume of cytoplasm and which is not visibly associated with blood vessels were counted in the area. Then, the cell number per mm^2 per case was calculated. At least 10 randomly selected fields were analyzed for each specimen.

Proliferating cells nuclear antigen (PCNA), von Willebrand factor, CD31, VEGF, Dll4, Jag-1 were detected using routine indirect immunohistochemical staining (Gunin et al., 2005, 2011). Slides were also preincubated in 0.1% hydrogen peroxide in distilled water for 10 min to block endogenous peroxidase activity. Polyclonal rabbit anti-PCNA antibody (AHP1419, AbD Serotec, Oxford, UK) diluted 1:100, polyclonal mouse anti-human von Willebrand factor antibody (M 0082, DakoCytomation, Glostrup, Denmark) diluted 1:100, monoclonal mouse anti-human CD31 antibody (M 0823, DakoCytomation, Glostrup, Denmark) diluted 1:50, polyclonal rabbit anti-human VEGF antibody (NBP1-22844, Novus Biologicals Inc., Littleton, CO, USA) prediluted by the manufacturer; polyclonal rabbit anti-human Dll4 antibody (AHP1274, AbD Serotec, Oxford, UK) diluted 1:50, and polyclonal rabbit anti-human Jag-1 antibody (NBP1-90208, Novus Biologicals Inc., Littleton, CO, USA) diluted 1:50 were used as primary antibody. Anti-mouse or anti-rabbit EnVision+ System conjugated with peroxidase (K4000, K4002, DakoCytomation, Glostrup, Denmark) was used as secondary antibody, and peroxidase activity was then revealed by the use of hydrogen peroxide and diaminobenzidine (Sigma Chemical Co., St. Louis, MO, USA) technique. Cell nuclei were counterstained with hematoxylin. Control sections were stained in a similar manner, except that the primary antibody was replaced with normal mouse or rabbit serum. There was no specific staining in control slides for all protocols applied.

To count for PCNA positive cells, blood vessels positively stained for von Willebrand factor or CD31, slides were initially photographed as mentioned above. Then, pictures were loaded in Sigma Scan Pro 5.0 software (SPSS Inc., Chicago, IL, USA), and areas of tissue for counting were selected and then measured (Gunin et al., 2004, 2011). For PCNA positive cell counting, PCNA positive and PCNA negative fibroblast-like cells were counted in the area. Then percent of PCNA positive cells was found by dividing the number of PCNA positive fibroblast-like cells into a total number fibroblast-like cells counted. For von Willebrand or CD31 positive blood vessel counting, positively stained blood vessels were counted in a previously measured area of dermis. Then, the number of blood vessels per mm^2 per case was calculated. At least 10 randomly selected fields were analyzed for each specimen.

Intensity of immunohistochemical staining of blood vessels for VEGF, Dll4 and Jag-1 was estimated by microscopic examination of slides by two investigators independently. Results were scored as absent, weak, middle and strong intensity of staining. Then, percent of each type of intensity of staining was calculated for each age-group.

There were 359 specimens available (199 men, 160 women) for fibroblast-like cell counting. There were 139 specimens available (104 men, 35 women) for PCNA positive cell counting. There were 92 specimens available (46 men, 46 women) for von Willebrand positive blood vessel counting. There were 94 specimens available (54 men, 40 women) for CD31 positive blood vessel counting. There were 128 specimens available (69 men, 59 women) for VEGF positive blood vessel analysis. There were 150 specimens available (78 men, 72 women) for Dll4 positive blood vessel analysis. There were 120 specimens available (62 men, 58 women) for Jag-1 positive blood vessel analysis.