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Enhanced Proliferation and Altered Intracellular Zinc Levels in Early- and Late-Passage Mouse Aorta Smooth Muscle Cells

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Abstract Cell growth and DNA synthesis were studied from a cultured early- and late- passage mouse aorta smooth muscle cell (MASMC) because the proliferation of vascular smooth muscle cell (VSMC) is a key factor in development of atherosclerosis. In this study, the cells were cultured in fetal bovine serum (FBS) and stimulated by growth factors such as thrombin and platelet-derived growth factor-BB (PDGF-BB). Compared to the number of early-passage MASMC (passage 3 to 9) the number of late-passage MASMC (passage 30 to 40) in a normal serum state was increased 2 fold at Day 1, 3 and 6 in culture, respectively. Incorporation of [^{3}H] thymidine into DNA induced by serum, PDGF and thrombin in late-passage MASMC was greater than those in early-passage MASMC. We also examined whether intracellular zinc levels would be an aging factor or not. The intracellular zinc level in early- and late-passage MASMC was monitored by using the zinc probe dye *N*-(6-methoxy-8-quinolyl)-*p*-toluenesulfonamide. It is interested that late-passage MASMC increased the intracellular fluorescence level of zinc, more than the early passage MASMC did. The alterations of intracellular zinc level occur concurrently with changes in MASMC proliferation rate during aging. This data suggest that the age-associated changes in zinc concentrations may provide a new *in vitro* model for the study of smooth muscle cell differentiation.

Keywords: atherosclerosis, proliferation, mouse aorta smooth muscle cells (MASMC), zinc, aging

INTRODUCTION

Earlier studies have shown that smooth muscle cells of different ages differ markedly in their *in vitro* growth characteristics. Vascular smooth muscle cell (VSMC) cultures obtained from humans and rats have been used widely as a model of VSMC proliferation in arterial wall [1-3]. Arterial VSMC have a high proliferative activity during fetal and neonatal life, whereas the rate of DNA replication is very slow in the adult artery [4]. In smooth muscle cells, cytoskeletal proteins are believed to play an important role in determining cell shape and phenotype as well as morphogenetic movements [5,6]. The contractile phenotype of VSMC has a primary role in controlling vessel tone [7]. Alterations in phenotype resulting from vascular injury influences migration and proliferation of these cells [8]. The conversion of VSMC from the embryonic to the late fetal/adult growth phenotype has an important component of a developmental system controlling vascular morphogenesis [9]. But, to date, to our knowledge, the difference between early- and late-passage VSMC has not been studied in detail, especially in mouse. It is well known that aging is an independent risk factor for several cardiovascular diseases [10]. Numerous studies suggest that reactive oxygen species (ROS) may also participate in aging [11-13]. Consistent with a role for ROS in senescence, examination of cells in culture suggests that older cells have higher levels of ROS than younger ones [14]. Some investigators reported that zinc induced accumulation of toxic free radicals, leads to neuronal apoptosis and necrosis [15,27]. In non-neuronal cells, zinc was also shown to trigger a formation of superoxide and hydrogen peroxide in adipocytes [16]. However, in cultured HL-60 cells and neonatal mice cardiomyocytes, metallothionein were induced by zinc and was shown to protect the cells from H_2O_2 toxicity [17,18]. Recently, we have successfully cultured smooth muscle cells from mouse aorta. Using this *in vitro* cellular system, we have studied age-associated cellular proliferation changes of MASMC such as cell growth and DNA synthesis. Based on the recent study related to zinc concentrations to aging in neuronal system [19], we suggest the possibility that vascular smooth muscle cells may induce replicative senescence by altering the intracellular levels of zinc.

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MATERIALS AND METHODS

Cell Culture

Mouse aortic smooth muscle cells (MASMC) were harvested from male mouse by enzymatic dissociation according to the modified method of Gunther *et. al.* [20]. MASMC was cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO, Grand Island, NY, U.S.A.) and supplemented with 10% fetal bovine serum (FBS, GIBCO, Grand Island, NY, U.S.A.), penicillin (100 units/mL) and streptomycin (100 g/mL) (GIBCO, Grand Island, NY, U.S.A.). Cells were passaged in every 3-5 days, and early-passage cells (passage 3-9) and late-passage cells (30-40) were routinely used for each experiment.

Growth Curve

Early- and late-passage cells were seeded in 100-mm petri dishes at a number of 1×10^4 cells. These cells were fed with fresh Dulbecco's modified Eagle's medium (DMEM, GIBCO, Grand Island, NY, U.S.A.) twice a week. On Day 1, 3 and 6, the cells were harvested after treatment of 0.25% trypsin-EDTA solution. The cell suspension was mixed with an equal amount of 0.4% trypan blue stain (GIBCO, Grand Island, NY, U.S.A.). The mixture of cell suspension and dye were placed in a hematocytometer and the cell number was obtained by counting the stain-resistant cells under a converted light microscope. Three dishes per group were used to determine a cell number at each time point and each sample was counted in triplicate. The experiment was performed using cells from three different preparations. The average cell number was plotted against the number of days in culture and the resulting curve was referred to as the growth curve.

Determination of DNA Synthesis

The growth responses to chemical stimulation were studied at early-passage and late-passage cells. DNA synthesis induced by thrombin, FBS, and platelet-derived growth factor-BB (PDGF-BB) (R&D Systems Inc., Minneapolis, MN, U.S.A.) was assessed by measuring the incorporation of [methyl-^{3}H] thymidine into DNA fragments. The cells were seeded at a field density of $1 \times 10^4/cm^2$ into 24-well culture dishes in DMEM containing 10% FBS and allowed to attach for 24 h. The culture medium was replaced by DMEM medium containing 0.1% FBS for 48 h so that the cells became quiescent [21]. After washing the wells, the cells were exposed to growth stimulants (1 U/mL thrombin, 10 ng/mL PDGF-BB, and 10% FBS) for an additional 20 h and were subsequently pulsed with 1 μCi/mL [methyl-^{3}H] thymidine (Dupont/NEN) for 4 h. The cells were washed with PBS and fixed in 10% trichloroacetic acid after labeling and then washed with 95% ethanol. Incorporated [^{3}H] thymidine was extracted in 0.2 M NaOH and measured in a liquid scintillation counter as previously described [22].

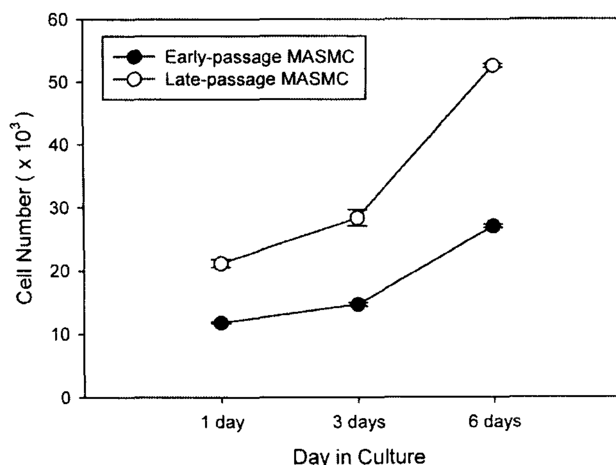

Fig. 1. The proliferation of cultured early-passage and late-passage MASMC after stimulation by 10% FBS.

TSQ Staining

The intracellular Zn^{2+} was visualized using *N*-6-methoxy-8-quinolyl-*p*-toluene sulfonamide (TSQ) (Molecular Probes, Eugene, OR, U.S.A.), a membrane permeable Zn^{2+}-chelating dye, as described by Frederickson *et al.* [23]. In brief, cultures were ashed with HCSS and incubated in HCSS containing 0.01% TSQ. After 5 min cultures were observed under fluorescence microscopy with an UV filter (excitation 365 nm, dichroic 400 nm, barrier 450 nm).

RESULTS AND DISCUSSION

Early- and late-passage MASMC were counted on Day 3, 7 and 14 in culture and a growth curve was constructed as shown in Fig. 1. As early as 3days in culture, late-passage MASMC displayed a faster growth rate than early-passage MASMC. This trend continued on Day 7 and 14 in culture. [^{3}H] thymidine incorporation of cultured early- and late-passage MASMC after stimulation by growth stimulants are shown in Fig. 2. In both early- and late-passage MASMC, [^{3}H] thymidine incorporation was induced by 10% serum stimulation. In late-passage MASMC was 2 times greater than in early-passage MASMC. The mitogenic activities of PDGF-BB and Thrombin were monitored by [^{3}H] thymidine incorporation.

Incorporation of [^{3}H] thymidine into DNA in late passage MASMC were significantly higher, compared with those in early-passage MASMC. To examine whether intracellular level of zinc is related to the aging process or not, both early- and late-passage MASMC were stained with the TSQ, a zinc specific probe. Intracellular TSQ fluorescence staining in cultured early- and late-passage MASMC are shown in Fig. 3. Even though we didn't treat any reagents and chemicals, late-passage MASMC in culture significantly increased intra cellular levels of zinc (Fig. 3(d)), the early-passage MASMC

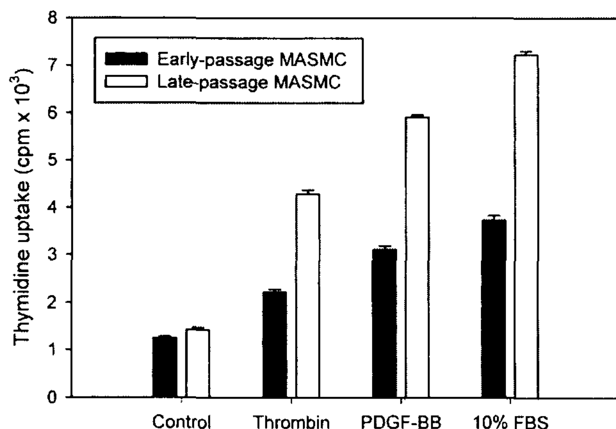

Fig. 2. [^{3}H] thymidine incorporation of cultured early-passage and late-passage MASMC after stimulation by growth stimulants.

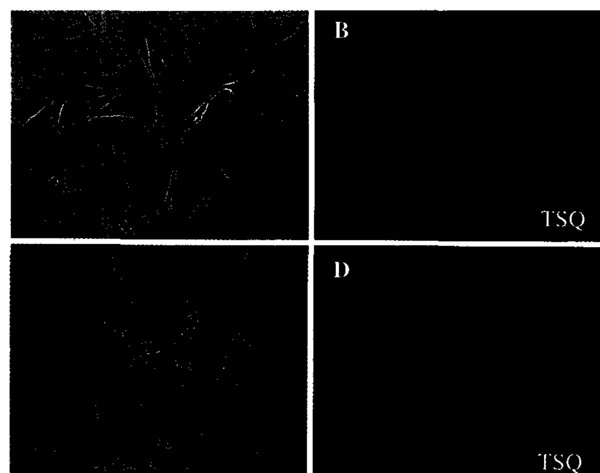

Fig. 3. Intracellular TSQ fluorescence staining in cultured early-passage and late-passage MASMC [Bright fields (left) and TSQ fluorescence staining (right panel) photomicrographs in vascular smooth muscle cells culture for 24 h.

was not (Fig. 3(b)). We also found a morphological change between early- and late-passage MASMC (Fig. 3(a), (c)). Vascular smooth muscle cell (VSMC) cultures obtained from rats and humans have been used widely as a model of VSMC proliferation in aging [1-3]. Fetal, neonatal VSMC and adult VSMC have been used in these experiments [1-4]. But, to date, in our knowledge, the difference between early- and late-passage VSMC has not been shown. Late-passage MASMC in culture exhibited higher rates of cell growth and DNA synthesis as mentioned above. Our results demonstrate phenotypic modulation of MASMC during aging may be related to alterations in proliferation. Since age-related vascular remodeling is characterized mainly by smooth muscle hyperplasia and intimal thickening associated with VSMC migration [24], the *in vitro* data in the present study would be valuable to the investigation of age-related remodeling in the vasculature. The cardiovascular system is influenced by the natural presence of reactive oxygen species (ROS), especially during aging. Many researchers have looked at the free radical theory of aging as the most accepted hypothesis of cellular senescence [25] which is induced by altering the intracellular levels of ROS [26]. Recently, some studies have suggested that zinc exposure induces the generation of intracellular ROS related to cell death [15,27]. In contrast, other studies have reported that metallothionein-over-expres-sing neonatal mouse cardiomyocytes are resistant to H_2O_2 toxicity which are induced by zinc [16]. Alternatively, it remains to be unknown whether zinc plays a role in prooxidant or antioxidant. Our findings suggest that increased intracellular zinc levels in aging cells are consistent with increased age-related proliferation of MASMC. Further studies are needed to delineate the mechanism of age-related zinc accumulation. As described in this study, this accumulation of zinc level is likely to be an important factor in combating human aging.

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