

Hypertension Decreases Replication Potential of Arterial Smooth Muscle Cells: Aortic Coarctation in Humans as a Model (41070)

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Abstract. Human arterial smooth muscle cells cultured from tissue proximal to the site of aortic coarctation had fewer cumulative population doublings and a slower replication rate than cells grown from tissue distal to the coarctation. Since cells proximal to the coarctation presumably had been stimulated to divide excessively *in vivo* by chronic exposure to elevated intraarterial pressure, these results using a unique human experimental model of hypertension suggest that the number of prior cell divisions limits their further replicative potential. These characteristics of accelerated aging induced by chronic exposure to hypertension may have relevance to the pathogenesis of atherosclerosis.

The replication potential of arterial smooth muscle cells may play a role in the pathogenesis of atherosclerosis, since proliferation of these cells appears to be a key feature of this disease process (1). There is some evidence that the number of previous replications of diploid cells *in vivo* influences subsequent cell replication potential *in vitro*. For example, in *in vitro* lifespan (cumulative population doublings) of cultured human skin fibroblasts and arterial smooth muscle cells appears to be related inversely to the chronological age of the donor (2-4). With cultured fibroblasts, a linear decline in replicative potential as a function of donor age was demonstrated (5, 6).

Hypertension *in vivo* stimulated the proliferation of arterial smooth muscle cells residing in thickened muscular arteries in areas subjected to increased arterial pressure (7). In coarctation of the aorta, a congenital constriction of the thoracic aorta found near the site of the entrance to the fetal ductus arteriosus, blood flow is obstructed. When the obstruction is sufficiently severe, a pressure gradient is produced across the coarctation and collateral circulation develops, bypassing the narrowed area. Hypertension usually is found proximal to the coarctation. Distally, mean blood pressure remains within the normal

range but the pulse pressure is narrowed (8). Unless the coarctation is surgically repaired, atherogenesis progresses at an accelerated rate in the hypertensive portion of arterial tree and affected individuals are prone to develop early coronary artery and cerebral vascular atherosclerosis (9). Since the coarctation and straddling cuffs of aortic tissue are excised at surgical repair, the specimen provides aortic tissue with areas exposed to elevated as well as to lower levels of pressure. Thus, aortic coarctation provides a unique human experimental model to test whether the increased number of previous replications of arterial smooth muscle cells, stimulated by prolonged exposure to high arterial pressure, diminishes cell replication potential since the same piece of tissue can be used to provide explants from both sides of the obstruction.

Methods. Aortic specimens were obtained from 13 patients, ages 0.5 to 32 years, undergoing coarctectomy [proximal phasic blood pressure, 130/70-190/98 mm Hg; distal mean blood pressure, 55-79 mm Hg]. Explants of intimal-medial segments of arterial tissue from the proximal (hypertensive) and distal (control) sides of the coarctation were prepared according to the methods of Ross (10, 11) to provide homogeneous cultures of smooth muscle cells. Typically the proximal side of the coarcted aorta was thicker than the distal side, presumably reflecting the proliferation and hyperplasia of vascular smooth muscle

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cells produced by hypertension (12–14). The homogeneity, growth characteristics, and morphology of arterial smooth muscle cells cultured by this technique have been described previously (10, 11, 15).

The explants were transferred into 25-cm² plastic flasks (Falcon), using a minimal amount of medium sufficient to just cover the explants and promote adherence to the plastic surface. Approximately twenty to thirty 1.0-mm² size explants were seeded into each flask. The medium used was modified Dulbecco–Vogt medium with glucose added to a final glucose concentration of 300 mg/dl and containing 10% fetal bovine serum (Gibco). All flasks were incubated at 37° in a humidified atmosphere of 95% air–5% CO₂. After several days, sufficient to allow the explants to adhere to the bottom of the flask, medium was added gradually, increasing to a final volume of 4 ml. The latent period for cellular outgrowth from the explants was similar from proximal and distal sides. When sufficient cells (usually 5×10^5 or greater) had grown out, the cultures were trypsinized with 1 ml of 0.5% trypsin and transferred into larger 75-cm² plastic flasks, containing 10 ml of medium. Cells were then fed twice weekly by replacement of approximately two-thirds of the medium in the flask with fresh medium. These cells were harvested approximately every 8 weeks by trypsinization, seeding 5×10^5 cells in recipient 75-cm² flasks where possible. The inoculum necessarily was reduced when replication rate slowed. This procedure was repeated until the cells failed to replicate, using the criterion of two consecutive trypsinizations in which cell number at harvest was less than cell number of inoculum. The number of cell doublings was calculated by the formula $N = X_0 2^n$ where N is the final population number of cells, X_0 is the number of cells in the inoculum, and n is the number of generations. Since the number of cells growing out of the original explants to initiate the population doublings was unknown, it was assumed arbitrarily that all cell strains started with 1×10^5 cells. Thus, the number of population doublings prior to the first trypsinization of the flask containing

the explants was added to the total number of subsequent doublings to obtain the final estimate of the cumulative population doublings for each cell strain. Plating efficiency 6 hr after subculture averaged $55 \pm 14\%$ (SD) for early passage arterial smooth muscle cells obtained from the hypertensive segment, and $57 \pm 14\%$ for cells from the normotensive segment.

Results and Discussion. The cumulative population doublings of smooth muscle cells obtained from the proximal aortic tissue exposed to increased arterial pressure and from the distal control aortic tissue straddling the coarctation were compared in the 12 specimens that were successfully carried for more than two passages. Cultured arterial smooth cells from tissue proximal to the coarctation tended to have shortened *in vitro* lifespans compared to those from tissue distal to the coarctation. A significant correlation was found between donor age and the shortening of *in vitro* lifespan ($r = 0.68$, $P < 0.01$, $n = 12$). All donors more than 6 years old showed a decrease in total population doublings of cells cultured from the hypertensive side (mean decrease 31%, $P < 0.01$; $n = 6$, Table I). These results lend support to the suggestion that the number of previous replications of arterial smooth muscle cells *in vivo* determine cell replication potential in culture.

Furthermore, since lifespan of cultured human arterial cells decreases as a function of donor age over an 80-year period (16), this finding suggests that prolonged hypertension accelerates *in vivo* aging of arterial smooth muscle cells. A minimum time of exposure to high pressure seems to be required for its effect on cell lifespan to become apparent.

Additional experiments were performed to test whether the proliferation rate of cells cultured from the hypertensive side of the coarctation was affected by *in vivo* exposure to increased arterial pressure. During early passage, similar numbers of cells from the proximal and distal sides of the coarctation were plated in parallel in 35-mm plastic petri dishes using the medium described and an atmosphere of 5% CO₂, 90%

TABLE I. LIFESPAN AND GROWTH RATE OF ARTERIAL SMOOTH MUSCLE CELLS CULTURED FROM PROXIMAL AND DISTAL SIDES OF AORTIC COARCTATIONS

Donor age (yrs)	In vitro lifespan			Growth rate		
	Total doublings		% Diff. ^a	Doublings/day		% Diff. ^a
	Proximal	Distal		Proximal	Distal	
0.5	5.8	4.0	45.0	—	—	—
4.8	7.2	7.3	-1.4	—	—	—
4.9	3.7	3.3	12.1	0.100	0.229	-56.3
5.5	11.5	10.9	5.5	—	—	—
5.6	9.2	13.2	-30.3	0.235	0.282	-16.7
5.6	8.3	5.0	66	—	—	—
6.4	10.0	13.9	-28.1	0.219	0.232	-5.6
8	4.5	6.6	-31.8	0.104	0.242	-57.0
9	3.6	6.9	-47.8	0.163	0.203	-19.7
11.1	6.9	9.1	-24.2	—	—	—
13.1	4.6	5.5	-16.3	0.209	0.165	26.7
17.2	—	—	—	0.089	0.221	-59.7
19.2	6.9	11.0	-37.3	0.293	0.370	-20.8
32	—	—	—	0.237	0.236	0.4

^a $(P - D)/D \times 100$.

N₂, 5% O₂. This atmosphere was used since we have previously shown that enhanced proliferation rates of cultured human arterial smooth muscle cells can be obtained when they are grown in an atmosphere of lower oxygen concentration than that present in room air (17). This culture condition appeared to amplify the difference in replication rate between cells from the proximal and distal segments. After 6, 24, 48, and 72 hr, the cell number was determined in quadruplicate by counting in a Fuchs-Rosenthal hemocytometer an aliquot of suspended cells released from each dish by trypsinization. The replication rate during the logarithmic growth phase was calculated by the least-squares method and expressed as the increase in cell number during the 3-day period starting with 6 hr, a time at which the number of inoculated cells attached to the dish can be used as baseline.

Cells cultured from the proximal (hypertensive) segment showed a significantly decreased proliferation rate compared to those from the distal (control) segment at all ages (23% decrease; $P < 0.05$) (Table I). Seven out of nine specimens demonstrated this effect of *in vivo* hypertension on arterial cell growth rate. Decreased cell repli-

cation rates in culture are also characteristic of *in vivo* aging. Thus, both decreased *in vitro* lifespan (cumulative population doublings) and decreased *in vitro* replication rates were demonstrated for arterial smooth muscle cells cultured from that portion of the coarcted aorta exposed to chronically elevated intraarterial pressure. These characteristics of accelerated cell aging may have relevance to atherogenesis, which is known to increase exponentially with aging in humans (18). Thus, hypertension, by chronically stimulating arterial smooth muscle cell proliferation *in vivo* (12-14), may be related to atherogenesis, in part by decreasing residual replication potential, which may be a mechanism similar to that associated with aging.

These results also support the concept that a critical limit of divisions of human diploid cells determines their replicative lifespan. Harley and Goldstein (19) found that in circular outgrowths of cultured human skin fibroblasts, cells at the circumference, which divide actively, consume more of their replicative lifespan than cells located more centrally, which tend to be density inhibited. In addition, it has recently been shown that skin fibroblasts obtained from the habitually sun-exposed lateral aspect of

the arm of male donors had fewer cumulative population doublings than did paired cell strains obtained from the nonexposed medial aspect (20). Our results extend these findings to arterial smooth muscle cells that have been stimulated to divide excessively *in vivo* by chronic exposure to elevated intraarterial pressure, using a unique human experimental model of hypertension with a built-in control, coarctation of the aorta.

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