Constitutive Activation of rac1 Results in Mitochondrial Oxidative Stress and Induces Premature Endothelial Cell Senescence

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Objective—Oxidative stress has been implicated in cellular senescence and vascular aging. We determined the role and mechanism of the small GTPase rac1 in vascular endothelial cell senescence.

Methods and Results—Adenoviral-mediated expression of the constitutively active allele of rac1 (rac1V12) in human umbilical vein endothelial cells resulted in mitochondrial oxidative stress with induction of biochemical, molecular, and morphological features of senescence. Suppression of mitochondrial oxidative stress abrogated rac1-induced premature senescence. Rac1V12 expression also resulted in an increase in endothelial ceramide levels. Moreover, premature endothelial cell senescence induced by an exogenous cell-permeable ceramide analog was not suppressed by inhibiting endogenous rac1 signaling. Finally, in human umbilical vein endothelial cells that had undergone replicative senescence, rac1 was not activated, and expression of the dominant-negative rac1 allele (rac1N17) did not suppress mitochondrial oxidative stress.

Conclusions—These findings paint a picture in which the constitutive activation of rac1o in vascular endothelial cell senescence.

Key Words: endothelium ■ rac1 ■ oxidative stress ■ senescence ■ mitochondria

Advanced age is the single most important risk factor for the development of atherosclerosis. Vascular aging in humans and animals is associated with an increase in the levels of reactive oxygen species (ROS), leading to oxidative stress. Replicative senescence, the in vitro correlate of organismal aging, is also associated with an increase in intracellular ROS levels, and measures that decrease oxidative stress result in prolongation of cellular and organismal lifespan.

A significant body of evidence has accumulated regarding the molecular mechanisms that regulate ROS production in cells, and senescent cells in particular. In human diploid fibroblasts, expression of an activated allele of the protooncogene c-ras results in premature senescence through induction of oxidative stress. Ras is not unique among the small GTPase superfamily in its ability to regulate ROS production. Importantly, some of the phenotypes that c-H-ras promotes, such as cellular transformation, are mediated through rac1, another member of this family. Rac1 is a regulatory component of the plasma membrane NAD(P)H oxidase(s) that are responsible for the production of intracellular ROS in a wide variety of cell types. However, proof that rac1 or rac1-regulated ROS are involved in cellular aging or premature senescence is lacking. We hypothesized that constitutive activation of rac1, by inducing oxidative stress, plays an important role in determining the senescent phenotype. To test this hypothesis, we used primary human umbilical vein endothelial cells (HUVECs), a validated model of cellular senescence, and one that may bear relevance to vascular aging in vivo.

Methods

Cell Culture

HUVECs were purchased from Clonetics and were grown and maintained in endothelial growth medium. In growth experiments, 1 × 10^4 cells were sparsely seeded, allowed to grow with addition of fresh medium every 48 hours, and counted on the specified days. To achieve replicative senescence, cells were seeded sparsely, allowed to grow, and serially passaged. Population doublings (PDLs) were estimated based on seeding density and cell number. Monitoring of cell morphology showed that cells achieved senescence at a high PDL of approximately 22 to 30. Low passage cells (PDL < 15) were chosen as nonsenescent cells.

Adenoviruses

All viruses were constructed and used as previously described. AdDl312 encodes an E1-deleted adenovirus without a transgene, Adrac1V12 encodes the myc-tagged constitutively active allele of rac1, and Adrac1N17 encodes the myc-tagged dominant-negative allele of rac1. Viruses were propagated in HEK 293 cells, purified on double cesium gradients, and tittered. Expression of the rac1 alleles was detected with a myc antibody (Santa Cruz Biotech).
Senescence-Associated Acidic β-Galactosidase Activity (SA-β-Gal)
SA-β-gal-positive cells were identified as previously described.10 Cells were fixed in 0.2% glutaraldehyde in PBS and incubated for 16 hours with X-gal staining solution: 1 mg of 5-bromo-4-chlor-3-indolyl-β-D-galactopyranoside, 5 mmol/L potassium ferrocyanide, 5 mmol/L potassium ferricyanide, 2 mmol/L MgCl₂, pH 6.0. Cells with cytosolic blue staining were counted from four random fields with at least 100 cells/field, and the percentage of SA-β-Gal-positive cells was calculated.

Northern Blot Analysis
Total cellular RNA was extracted by a standard protocol, electrophoresed, and hybridized with a 32P-labeled single-strand DNA probe of human plasminogen activator inhibitor-1 (PAI-1)11 or 18S rRNA. Quantitative values are expressed after normalization with 18S.

Mitochondrial Oxidative Stress
Mitochondrial ROS were measured with the mitochondrial-specific, redox-sensitive fluorophore dihydorhodamine 123 at 5 μmol/L as previously described.9

H₂O₂
H₂O₂ was quantified using a fluorescence-based assay (Amplex Red; Molecular Probes) as previously described.12

Rac1 Activity
Rac1 activity was measured with a p21-activated kinase 1 pull-down assay (UBL).

Apoptosis
Apoptosis was quantified with a colorimetric ELISA that detects cytoplasmic histone-DNA fragments (Boehringer-Manheim)

Ceramide Measurement
Cellular lipids were determined by the Diglyceride (DG) kinase assay.13 The products of DG kinase reaction were separated by thin-layer chromatography, and ceramide 1-(32P)-phosphate was identified by comigration with phosphorylated C18-ceramide standard and quantified by densitometry. Levels of ceramide were normalized for the final cell number in each condition. Ceramide content per 10⁵ cells was determined by quantitative comparison with the C18-ceramide standard.

Telomerase
Telomerase activity was detected with a TRAPEZE Telomerase Detection kit (Intergen Corp.) using isotopic detection of telomerase repeat amplification protocol products.

Statistics
Data are expressed as mean±SD from at least 2 independent experiments. Statistical analysis was performed by Student t test.

Results
We first examined ROS levels, and the source of such ROS, in endothelial cells in which rac1 signaling was activated. To accomplish this, HUVECs were infected with an adenovirus encoding the constitutively active allele of rac1 (rac1V12). Such cells displayed significant expression of rac1V12 (Figure 1A, inset), and a marked increase in mitochondrial ROS (mitochondrial oxidative stress; Figure 1A), as judged by the mitochondrial-specific redox-sensitive fluorophore DHR123, when compared with HUVECs infected with the null virus AdDl312. Similar results were obtained when oxidative stress was measured with another assay that specifically detects hydrogen peroxide (H₂O₂; Figure 1B). Moreover, treatment with apocynin, an inhibitor of the plasma membrane NAD(P)H oxidase, did not suppress the rac1V12-stimulated increase in H₂O₂ levels (Figure 1B). In contrast, treatment with rotenone, a specific inhibitor of site I of the mitochondrial electron transport chain, suppressed the rac1V12-induced mitochondrial oxidative stress (Figure 1C). In addition, to manipulate mitochondrial ROS levels, we exposed rac1V12-expressing cells to hypoxia (3% O₂). This treatment resulted in a 32% reduction of rac1V12-induced mitochondrial oxidative stress (Figure 1D). These findings show that constitutive activation of rac1 in endothelial cells results in mitochondrial oxidative stress that is independent of the plasma membrane NAD(P)H oxidase and that this increase is the result of disruption of the mitochondrial respiratory chain.

Ceramide, a lipid second messenger, has also been linked to disruption of the mitochondrial respiratory chain and induction of mitochondrial oxidative stress.14 In addition, exogenous ceramide analogues are capable of inducing the senescent phenotype in human diploid fibroblasts.15,16 Therefore, we were interested in first determining whether rac1 regulates ceramide levels in endothelial cells. Rac1V12-expressing cells displayed significant elevations in intracellular ceramid compared with control cells (Figure 2). This result implicates ceramide as a second messenger in rac1-induced mitochondrial oxidative stress.

We next sought to determine the role of rac1-induced oxidative stress in premature endothelial cell senescence. Compared with control AdDl312-infected cells, dose-dependent expression of rac1V12 resulted in a significant corresponding increase in the number of cells exhibiting SA-β-gal activity (Figure 3A). In addition to an increase in SA-β-gal activity, rac1V12-expressing endothelial cells also demonstrated an enlarged, flattened phenotype consistent with senescence (not shown) and growth arrest over a period of 1 week after viral infection compared with control uninfected and AdDl312-infected cells (Figure 3B). Finally, expression of rac1V12 resulted in induction of PAI-1, another marker of cellular senescence, when compared with control AdDl312-infected cells (Figure 3C). These findings demonstrate that constitutive activation of rac1 triggers rapid growth arrest and premature senescence of endothelial cells.

Because, under specific conditions and in certain cell types, expression of rac1V12 can induce apoptosis, we also determined the effect of rac1V12 expression on endothelial cell death. As compared with control cells, expression of rac1V12 did not induce apoptosis in HUVECs (Figure 3D). This finding shows that the suppression in cell number seen with rac1V12 expression is not caused by an increase in endothelial cell apoptosis.

To elaborate on the relationship between rac1 signaling and ceramide in the induction of endothelial cell senescence, we also examined the effect of inhibiting endogenous rac1 on ceramide-induced premature senescence. To this end, a dominant-negative allele of rac1 (rac1N17) was expressed in...
HUVECs using a recombinant adenovirus (Figure 3E, inset). Such cells exhibited no difference compared with control cells with respect to premature senescence induced by the cell-permeable ceramide analog C6-ceramide (Figure 3E). This finding reinforces that ceramide acts downstream of \( \text{rac} \) in the signaling pathway, leading to premature endothelial cell senescence.

We next determined the importance of mitochondrial oxidative stress in the development of the \( \text{rac} \)-induced senescent phenotype. \( \text{rac} \)-expressing cells were cultured in a 3% \( \text{O}_2 \) environment and SA-\( \beta \)-gal activity measured. In comparison with cells maintained in 20% \( \text{O}_2 \), lowering ambient oxygen to 3% resulted in a 38% decrease in \( \text{rac} \)-induced premature senescence (Figure 3F). Thus, premature endothelial cell senescence resulting from constitutive activation of \( \text{rac} \) is dependent on increase in mitochondrial oxidative stress.

In addition to establishing the importance of \( \text{rac} \) activation in the induction of premature cellular senescence, we were also interested in examining its role in replicative senescence. To this end, we determined the

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**Figure 1.** Mitochondrial oxidative stress induced by \( \text{rac} \) is suppressed by hypoxia, and rotenone. A, \( \text{rac} \) expression in HUVECs results in mitochondrial oxidative stress. Mitochondrial reactive oxygen species were detected by DHR fluorescence and quantified. *\( P < 0.05 \) compared with null AdDl312 virus. Inset: Western blot showing dose-dependent adenoviral expression of myc-tagged \( \text{rac} \). B, Increase in \( \text{H}_2 \text{O}_2 \) levels induced by expression of \( \text{rac} \) is not dependent on the plasma membrane NAD(P)H oxidase. \( \text{H}_2 \text{O}_2 \)-stimulated fluorescence was measured using the Amplex Red \( \text{H}_2 \text{O}_2 \) detection assay and are expressed as arbitrary fluorescence units. Apocynin was added at 200 \( \mu \text{mol/L} \) for 2 hours. *\( P < 0.05 \) compared with AdDl312. #\( P = \text{NS} \) compared with Ad\( \text{Rac} \). C, \( \text{rac} \)-induced mitochondrial oxidative stress is inhibited by treatment with the complex I mitochondrial electron transport inhibitor rotenone (5 \( \mu \text{mol/L} \) for 10 minutes). D, \( \text{rac} \)-induced mitochondrial oxidative stress is suppressed by growing cells under hypoxia (3% \( \text{O}_2 \) for 48 hours). *\( P < 0.05 \) compared with 20% \( \text{O}_2 \). Data are representative of at least 2 independent experiments.
activity of endogenous rac1 in HUVECs that had undergone replicative senescence through serial passaging. A significant proportion of cells that had undergone a high number of PDLs showed a senescent phenotype. However, in such cells, similar to nonsenescent cells, rac1 was not activated (Figure 4A). Moreover, inhibiting endogenous rac1 though adenoviral expression of a dominant-negative allele of rac1(rac1N17) did not suppress mitochondrial oxidative stress (Figure 4B). These findings demonstrate that endogenous rac1 activation is not important for the increase in mitochondrial oxidative stress during replicative senescence, underscoring the different mechanisms involved in the induction of premature and replicative senescence.

Discussion

Many previous reports have shown that rac1 regulates ROS production in a variety of cell types. Knowing the relationship between oxidative stress and senescence, it is therefore surprising that the literature is devoid of any direct examination of the role of rac1 in cellular aging. Our results are the first to show that constitutive activation of rac1 leads to growth arrest and premature cellular senescence. Moreover, in the context of endothelial cell biology, our findings are the first to demonstrate that rac1 regulates intracellular ceramide and mitochondrial ROS production. Taken together, these findings strongly implicate rac1-regulated ceramide production and mitochondrial oxidative stress in the induction of premature vascular endothelial cell senescence.

It would be fair to say that the effect of hypoxia on rac1V12-stimulated premature senescence does not conclusively demonstrate the causative role of mitochondrial oxidative stress in this phenomenon. Hypoxia may affect the generation of ROS by sources independent of the mitochondria, and as such can be considered a general antioxidant. Moreover, we cannot exclude the possibility that hypoxia, by suppressing oxidant production by one enzymatic source, may affect ROS generation by another.

It is also worth considering the commonality and differences with regards to mechanisms between premature and replicative endothelial cell senescence. Replicative endothelial cell senescence is associated with a decrease in telomer-
A Uninfected
AdDI312 200MOI
AdRac1V12 200MOI
GTP(active)- Rac1
Total Rac1
myc-rac1V12

B

PDL <15 >28 <15 <15

mitochondrial oxidative stress, likely have different operative mechanisms, sharing some common features, such as an increase in a marker

Figure 4. Rac1 does not regulate replicative senescence. A, Rac1 is not activated in HUVECs with replicative senescence. Rac1 activity in non-senescent (low PDL) and senescent (high PDL) HUVECs was determined with a p21-activated kinase pull-down assay. Infection with AdRac1V12 was used as a positive control. B, Rac1 does not regulate mitochondrial oxidative stress associated with replicative senescence. High-PDL HUVECs with a senescent morphology were infected with AdDI312 or AdRac1N17 and assessed for mitochondrial ROS using DHR fluorescence. *P<0.05 compared with AdDI312 PDL <28. #P=NS compared with AdDI312 PDL >28. Representative data from 2 independent experiments is shown.

Although this is the first report to directly ask whether activation of rac1 plays a causative role in premature cellular senescence in vitro, it is worth noting that rac1 expression is significantly upregulated in tissue from aged mice when compared with their young counterparts. In contrast to a possible role for rac1 in premature senescence and organismal aging, a recent report suggests that rac1 inhibits premature senescence of diploid fibroblasts induced by an active allele of H-ras (H-rasV12). However, the reduction in SA-β-Gal activity associated with rac1 expression reported in the study in question was small, and the effect of rac1V12 expression alone, independent of H-RasV12, on cell growth and senescence was not examined. Moreover, the expression level of rac1V12 was significantly lower than that which we achieved. Thus, the apparent contradictory results may be a reflection of the degree of expression of activated rac1, or could be the result of genuine differences in the phenotype induced by rac1V12 that are context specific and cell-type specific.

Along these same lines, a recent report has shown that expression of a constitutively active form of ras results in premature endothelial cell senescence. Although we did not examine the role of ras, based on previous evidence that rac1

Figure I, which can be accessed at http://atvb.ahajournals.org.

Nor did we find that endothelial cells that had undergone replicative senescence showed any increase in rac1 activity (Figure 4A). Moreover, the mitochondrial oxidative stress associated with replicative senescence was not suppressed by inhibiting endogenous rac1 signaling (Figure 4B). These observations reinforce the findings of previous studies that replicative and premature senescence, though sharing some common features, such as an increase in mitochondrial oxidative stress, likely have different operative mechanisms.

Acidic β-galactosidase activity is caused by an increase in lysosomal content. Although one biomarker of cellular senescence, it may also be a reflection of increased lysosomal activity responsible for the degradation of adenovirus. This was reflected in our finding that infection of HUVECs with the null AdDI312 virus resulted in an increase in SA-β-gal activity compared with uninfected cells (Figure 3A). However, this did not translate into a decrease in proliferative capacity or growth arrest (Figure 3B). Moreover, infection with Adrac1V12 led to a marked increase in multiple independent markers of senescence compared with cells infected with AdDI312. Taken together, these findings strongly suggest that although adenovirus infection does increase SA-β-gal activity in HUVECs, the effect seen with rac1V12 expression is a true indication of senescence.

The precise relationship between ceramide and proteins of the ras superfamily, in particular the rho GTPases, remains controversial. Some reports have shown that ceramide production is regulated by rho proteins, whereas others suggest that ceramide results in rac1 activation. Our observation that rac1N17 expression did not inhibit C6-ceramide-induced premature senescence coupled with the finding that expression of rac1V12 induced ceramide production suggests that in endothelial cells ceramide is a mediator, rather than an upstream regulator, of rac1-stimulated premature senescence, or that rac1 activation by ceramide may be dispensable for ceramide-induced endothelial cell senescence.

In the context of both senescence and apoptosis, ceramide has been implicated in inducing mitochondrial oxidative stress. Ceramide-stimulated disruption of the mitochondrial electron transport chain and consequent oxidative stress can be suppressed by inhibitors of both mitochondrial complex I and II. That rac1-induced mitochondrial oxidative stress was abrogated by rotenone (an inhibitor of complex I) is consistent with these findings and supports the hypothesis that ceramide mediates the effects of rac1 on mitochondrial ROS generation. Interestingly, despite increasing ceramide and mitochondrial ROS levels, rac1V12 did not induce endothelial cell apoptosis. In fact, most reports implicate rac1 in antiapoptotic signaling. This may be explained by the fact that in all our experiments endothelial cells were maintained in optimal growth conditions, and studies that have suggested that rac1 and rac1-regulated ceramide production are proapoptotic have been performed with an external apoptotic stimulus, such as serum and growth factor withdrawal.

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lies downstream of ras in stimulating the production of ROS,\textsuperscript{17} we would expect that rac1\textsuperscript{12} induced premature endothelial cell senescence is independent of endogenous ras activity.

In summary, we report a novel role for rac1 in the regulation of mitochondrial ROS and show that rac1-regulated mitochondrial oxidative stress leads to premature endothelial cell senescence. The in vivo physiological relevance of these findings remains to be determined. However, it is interesting to note that rac1 is activated by many inflammatory agents that have been implicated in the pathogenesis of atherosclerosis.\textsuperscript{29,30} Furthermore, endothelial cell senescence is associated with clinically relevant vascular disease.\textsuperscript{31} Therefore, it is tempting to speculate that repeated activation of rac1 during vascular inflammation or injury may play a role in premature aging of the endothelium with associated development of vascular pathologies.

References

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