Increased Radiosensitivity and Radioresistant DNA Synthesis in Cultured Fibroblasts From Patients With Coronary Atherosclerosis

Mohammed A. Hannan, Fareed Khougeer, Zoheir Halees, Aly M. Sanei, Bashir A. Khan

Abstract Cultured skin fibroblasts from five patients with atherosclerosis who underwent coronary artery bypass graft surgery were compared with those from one ataxia telangiectasia (AT) homozygote, three AT heterozygotes, and five healthy subjects to determine their sensitivity to gamma radiation as determined by a colony survival assay. Fibroblasts from four of these patients were also compared with those from two AT homozygotes, two AT heterozygotes, and three healthy subjects to determine postirradiation \[^{3}H\]thymidine incorporation, indicating the levels of radioresistant DNA synthesis (RDS). On the basis of colony survival assay, after long-term irradiation (at low dose rate, ie, 0.007 Gy/min), fibroblasts from all five patients with atherosclerosis exhibited radiosensitivity that was intermediate between that of the healthy subjects and that of patients with the known radiosensitive syndrome AT. However, there was a considerable interstrain difference in the radiosensitivity of fibroblasts from patients with atherosclerosis, with their mean $D_{50}$ values (radiation dose resulting in 10% cell survival) varying between 2.3 and 6.2 Gy, whereas the mean $D_{50}$ values for the cells from the AT homozygote, AT heterozygotes, and healthy subjects were 2.0, 3.8, and 9.0 Gy, respectively. One of the patients with atherosclerosis showed cellular radiosensitivity quite similar to that of the AT homozygote, up to 2% to 10% of survival levels after short- (at a dose rate of 8 Gy/min) and long-term irradiation, respectively. The results of \[^{3}H\]thymidine incorporation showed an AT heterozygote-like RDS in fibroblasts from patients with atherosclerosis that appeared to be intermediate between that of AT homozygotes and that of healthy subjects, suggesting a partial deregulation of cell cycle in the patients with atherosclerosis. Overall, the results suggest that increased cellular radiosensitivity and/or altered cell cycle regulation may be associated with atherosclerosis. (Arterioscler Thromb. 1994;14:1761-1766.)

Key Words • atherosclerosis • radiosensitivity • cell cycle • ataxia telangiectasia

Cardiovascular diseases and cancer are the major causes of morbidity and mortality in many countries. Several studies have suggested that multifactorial processes are involved in the origin of these diseases. The evidence of monoclonality, somatic mutations, and a possible involvement of mutagen/carcinogen exposure in both atherosclerosis and carcinogenesis strongly indicated that at some stage the two diseases may follow a similar pathway and share common risk factors. This would, inter alia, imply that genetic factors that increase susceptibility to malignancies may also enhance the risk of developing atherosclerosis. Among various carcinogenic factors, ionizing radiation has almost conclusively been shown to be atherogenic. Therefore, it is expected that individuals with increased sensitivity to radiation would be prone to develop atherosclerosis. The inherited disorder ataxia telangiectasia (AT), an autosomal recessive syndrome that is highly susceptible to malignancies, is characterized by its cellular hypersensitivity to ionizing radiation (showing reduced cell survival) as well as radioresistant DNA synthesis (RDS) (indicating a lack of $G_1$ arrest). As normal cells usually show $G_1$ arrest and, thus, an inhibition of DNA synthesis after irradiation, RDS is believed to be an indication of cell cycle deregulation. Swift and Chase and Swift et al presented epidemiological data showing an increased rate of mortality for ischemic heart disease as well as for various malignant diseases among AT gene carriers (obligate heterozygotes). If the hypothesis linking the AT gene with predisposition to ischemic heart disease were valid, one would expect to find AT heterozygote-like characteristics in patients with atherosclerosis. However, no experimental studies have been carried out to examine the biological properties of the body cells from patients with ischemic heart disease to find any similarity with those from AT heterozygotes. Although cells from AT homozygotes are highly sensitive to both short-term (high dose rate) and long-term (low dose rate) irradiation, those from most of the AT heterozygotes show a moderately enhanced cellular sensitivity to chronic irradiation. We therefore studied cultured skin fibroblasts from five patients with atherosclerosis and five healthy subjects to compare their response to irradiation by the same protocols used to analyze radiosensitivity of AT heterozygotes. In addition, we examined the levels of postirradiation DNA synthesis inhibition in the same cells. Surprisingly, these studies showed moderately enhanced radiation sensitivity and RDS in the fibroblasts from all the patients with atherosclerosis. The levels of both radiosensitivity and RDS in patients with atherosclerosis and AT heterozygotes occupied an
TABLE 1. Clinical Information on Patients With Atherosclerosis Whose Fibroblasts Were Analyzed for Radiosensitivity and Postirradiation DNA Synthesis Inhibition

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age, y</th>
<th>Sex</th>
<th>Unstable Angina</th>
<th>Blood Pressure, mm Hg</th>
<th>Antihypertensive Medication</th>
<th>Cholesterol Level</th>
<th>Triglyceride Level</th>
<th>HDL Level</th>
<th>Diabetes Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>60</td>
<td>Male</td>
<td>+</td>
<td>100/70</td>
<td>+</td>
<td>Normal</td>
<td>Normal</td>
<td>Low</td>
<td>No</td>
</tr>
<tr>
<td>2</td>
<td>55</td>
<td>Male</td>
<td>+</td>
<td>104/65</td>
<td>+</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>No</td>
</tr>
<tr>
<td>3</td>
<td>60</td>
<td>Female</td>
<td>+</td>
<td>130/90</td>
<td>No</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Unknown</td>
<td>No</td>
</tr>
<tr>
<td>4</td>
<td>44</td>
<td>Female</td>
<td>Stable</td>
<td>140/90</td>
<td>+</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Moderate, left main, atypical chest pain after surgery</td>
</tr>
<tr>
<td>5</td>
<td>51</td>
<td>Male</td>
<td>+</td>
<td>110/70</td>
<td>+</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>NIDDM Severe</td>
</tr>
</tbody>
</table>

HDL indicates high-density lipoprotein; NIDDM, non-insulin-dependent diabetes mellitus.

intermediate position between those of AT homozygotes and healthy subjects. The results suggested that an AT heterozygote-like cell cycle abnormality combined with or leading to a moderate increase in cellular radiosensitivity may be associated with ischemic heart disease.

Methods

Cell Cultures

Cutaneous skin biopsies were obtained from both patients and healthy subjects (on consent). Primary fibroblast cultures were developed by growing the skin explants in minimal essential medium (MEM) supplemented with Earl's salts.
penicillin (100 U/mL), streptomycin (100 μg/mL), glutamine (2 mM), and 15% fetal bovine serum in 25-cm² tissue culture flasks incubated at 37°C in a humidified atmosphere with 5% CO₂/95% O₂.

Radiation Survival Analysis

Early passage (ie, 3 to 5) skin fibroblast cells from different subjects were grown in Ham's F12 containing the same supplements as in MEM. For long-term irradiation, confluent cell cultures in 100-mm dishes were irradiated inside a CO₂ incubator using a 137Cs source (Gamma Cell 1000, Atomic Energy of Canada Ltd) at a dose rate of 0.007 Gy/min. After irradiation, the growth medium was renewed, and the cells were left in the CO₂ incubator overnight. Cells from different irradiated plates were then trypsinized, harvested, diluted, and seeded (200 to 20 000 per plate) together with a feeder layer of 50-Gy radiation-inactivated normal human fibroblasts (60 000 per plate). After 3 weeks of incubation, with a weekly change of growth medium, macroscopic colonies (with more than 50 cells per colony) were scored as survivors. Phosphate-buffered saline was used to wash the cells, which were then stained with crystal violet to score the survivors. For short-term irradiation, late log phase cells were harvested, and 2-mL aliquots of suspension containing 2×10⁶ cells/mL taken into each of 15-mL tubes were exposed to gamma rays from the 137Cs source at a dose rate of 8 Gy/min. The procedures for cell plating and scoring survivors were the same as for long-term irradiation. Unirradiated cells were handled in the same manner for long- and short-term irradiation (except for irradiation) to serve as respective controls. Percent survival was calculated based on colony counts from at least four dishes for each radiation dose point compared with the respective control. The D₁₀ values (radiation dose resulting in 10% survival) were estimated from the survival curves without curve-fitting to compare the relative sensitivity of different cell strains.

Postirradiation DNA Synthesis Measurement

Log phase cells from five patients with coronary atherosclerosis, two AT homozygotes, two AT heterozygotes, and three healthy subjects were exposed to a single dose (4 Gy) of radiation. After 30 minutes of incubation, [³H]thymidine (specific activity, 5 μCi/mL) was added (1 μCi per dish) to the cells, which were incubated for an additional 2 hours. Cells were then washed three times with phosphate-buffered saline, trypsinized, and harvested on glass fiber filters using a skatron harvester before counting with a liquid scintillation spectrometer. Inhibition of DNA synthesis was expressed as percent of counts per minute in irradiated cell DNA compared with that in the unirradiated controls. The uptake of [³H]thymidine measured the level of postirradiation DNA synthesis per cell proliferation and thus indicated the level of RDS or cell cycle defect in cell strains from patients compared with the healthy subjects.

Results

A summary of the available clinical information on all five patients with atherosclerosis who underwent bypass surgery and were included in the present study is given in Table 1. There were two women (ages, 44 and 60 years) and three men (ages, 51, 55, and 60 years). Four of the patients (patients 1, 2, 3, and 5) had unstable
angina, and one patient (patient 4) had stable angina. Although four patients (1, 2, 4, and 5) were receiving antihypertensive medication, one patient (3) was not. Two of the patients (4 and 5) had non-insulin-dependent diabetes mellitus. The skin fibroblasts obtained from these patients and the healthy subjects (ages, 22 to 55 years) as well as an AT homozygote and an AT heterozygote were grown for several passages before their relative sensitivities to irradiation were analyzed.

The data on colony survival assay (CSA) illustrated in Fig 1 demonstrate the expected intermediate radiosensitivity of fibroblasts from the AT heterozygotes compared with the AT homozygote and the healthy subjects. The mean $D_0$ (radiation dose resulting in 10% survival) value was 2.0 Gy for the AT homozygote, 9 Gy for the healthy subjects, and 3.8 Gy for the AT heterozygotes. Fig 2 shows the survival curves obtained after long-term irradiation of fibroblasts from the five patients with atherosclerosis compared with those from the AT homozygote and the healthy subjects. The mean $D_0$ values (in Gy) were 2.3, 3.2, 4.1, 5.3, and 6.2, respectively, for patients with atherosclerosis 3, 4, 2, 5, and 1. Although the $D_0$ values varied considerably among the patients with atherosclerosis and differed from that obtained for the AT heterozygote, they occupied an intermediate position between those of the AT homozygote and the healthy subjects, indicating an enhanced cellular sensitivity of the patients to long-term irradiation relative to the healthy subjects.

Because long-term irradiation resulted in variable survival curves for the patients with atherosclerosis, with one (patient 3) showing a radiation response quite similar to that of the AT homozygote up to the 10% survival level, we also examined the cellular radiosensitivity of four patients compared with the healthy subjects and the AT homozygote after short-term irradiation. Although three of these patients were indistinguishable from the healthy subjects with respect to their response to short-term irradiation, cells from patient 3 again showed a surprising similarity (up to 2% survival level) to those of the AT homozygote in its enhanced radiosensitivity (Fig 3).

Because inhibition of DNA synthesis after irradiation is an indication of normal cell cycle regulation, we examined the levels of postirradiation $[^3H]$thymidine incorporation in fibroblast cells from four patients with atherosclerosis, two AT homozygotes, two AT heterozygotes, and three healthy subjects. As shown in Table 2, the percentage of DNA synthesis inhibition was 47±3 to

Fig 3. Short-term radiation survival curves of fibroblasts cells from the ataxia telangiectasia (AT) homozygote (c), four patients with atherosclerosis [patients 2 (a), 3 (m), 4 (c) and 5 (a)], and five healthy subjects (shaded area shows the range of survival). Bars for the AT homozygote and for each patient show the ± difference from the mean of two experimental values.
suggest that some or most of these patients may have been carriers of the AT gene, supporting the epidemiological observations of Swift and Chase. However, in view of the considerable interstrain differences in the level of radiosensitivity, particularly the response of patient 3, the possibility of other genes enhancing cellular sensitivity to genotoxic agents like radiation in patients with atherosclerosis cannot be ruled out. In this preliminary study, we also observed a moderate level of RDS in cells from the patients with atherosclerosis relative to those from the healthy subjects. A similar level of RDS was also observed in the AT heterozygotes. The AT gene has already been implicated in the regulation of cell cycle, and the cells from patients with AT have been shown to lack radiation-induced G1 arrest exhibiting a high degree of RDS. Thus, the data showing an intermediate level of RDS in the patients with atherosclerosis as well as the AT heterozygotes in this study might suggest that some of these patients were AT gene carriers. However, genes other than AT, particularly tumor suppressor genes like p53 and rb, have been shown to participate in the regulation of cell cycle. Any alteration in these genes would also affect the cell cycle progression after exposure to genotoxic agents and may, in fact, contribute to cellular hypersensitivity to these agents. Whether some or most of the patients with ischemic heart disease are carriers of the AT gene, as may be predicted from the epidemiological findings of Swift and Chase, can be confirmed only by using AT gene probes when they become available.

Meanwhile, the observation of increased cellular radiosensitivity and RDS in our preliminary study with a limited number of patients may stimulate further investigations on the role of inherent cell cycle defects in the pathogenetic events leading to atherosclerosis. Because radiation is a proven atherogen and various environmental radiomimetic agents, including free radicals, may play a part in the development of atherosclerotic plaques, the observation of enhanced cellular radiosensitivity in patients with atherosclerosis may be an indicator of genetic predisposition to this disease regardless of whether the radiosensitivity is due to the AT gene. Furthermore, the role of cell cycle control in the development of atherosclerosis should be the focus of further studies. It is known that smooth muscle cell proliferation on mitogenic stimulation is a major factor in atherosclerotic plaque formation. A few studies have recently analyzed the role of the rb gene in growth arrest in skeletal muscle cells and the genetic regulations of cell cycle and differentiation in vascular smooth muscle cells. The results of such studies suggest that cell cycle perturbed by mutations in genes like rb or p53 could greatly deregulate the mechanisms controlling smooth muscle cell proliferation in response to mitogenic stimulation. Individuals carrying such mutated genes may have an increased risk of developing atherosclerotic plaques. The carriers of mutations in tumor suppressor genes like p53 and rb as well as AT (alone or in combination) may be responsible for both altered cell cycle regulation and moderately increased cellular radiosensitivity in patients with atherosclerosis. Further studies on genes responsible for radiosensitivity and cell cycle defects in fibroblasts as well as smooth muscle cells from patients with atherosclerosis may provide a fascinating clue with respect to not only the genetic factors.

### Discussion

The data on CSA presented here demonstrate an enhanced sensitivity to long-term gamma irradiation of cultured skin fibroblasts derived from five patients with atherosclerosis. Although the degree of radiosensitivity considerably varied among the cell strains from these patients, their overall response to irradiation appeared to be intermediate between that of the radiosensitive syndrome AT (homozygotes) and the healthy subjects. The cells from patient 3 were found to be the most sensitive to both long- and short-term irradiation, whereas the remainder exhibited a moderately enhanced radiosensitivity only after long-term irradiation. The usefulness of long-term irradiation in detecting intermediate radiosensitivity of AT heterozygotes and body cells from patients with other diseases, including cancer, has been well documented. The demonstration of an intermediate radiosensitivity of skin fibroblasts from the patients with atherosclerosis might suggest that some or most of these patients may have

### Table 2. Postirradiation DNA Synthesis Inhibition In Fibroblasts From Four Patients With Atherosclerosis, Two AT Homozygotes, Two AT Heterozygotes, and Three Healthy Subjects, as Determined by [3H]Thymidine Incorporation

<table>
<thead>
<tr>
<th>Fibroblast Donor</th>
<th>DNA Synthesis Inhibition After 4 Gy of Gamma Irradiation, %*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients with atherosclerosis</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>30±7</td>
</tr>
<tr>
<td>3</td>
<td>32.5†</td>
</tr>
<tr>
<td>4</td>
<td>33±3</td>
</tr>
<tr>
<td>5</td>
<td>38±3</td>
</tr>
<tr>
<td>AT heterozygotes</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>32±3</td>
</tr>
<tr>
<td>2</td>
<td>33±2</td>
</tr>
<tr>
<td>AT homozygotes</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>13±3</td>
</tr>
<tr>
<td>2</td>
<td>13±8</td>
</tr>
<tr>
<td>Healthy subjects</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>48±3</td>
</tr>
<tr>
<td>2</td>
<td>47±3</td>
</tr>
<tr>
<td>3</td>
<td>47±3</td>
</tr>
</tbody>
</table>

*AT indicates ataxia telangiectasia; RDS, radiosensitive DNA synthesis. Values are mean±difference of two experiments. †Single experimental value.
predisposing to atherosclerosis but also the pathway shared by this disease and cancer.

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References

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