Involvement of protease activation in modulating radiation-sensitivity of human cells.

2nd Dept. of Biochemistry, Chiba Univ. Sch. Med., Chiba 260-8670, Japan

INTRODUCTION
Molecular mechanisms that supervise radiation-susceptibility of human cells are one of the important problems in Radiation Protection Science. We previously hypothesized that proteases may play a key role in cellular functions triggered by radiation as well as those in SOS functions of *E. coli* (1).

It has been found that the activity is detectable less than 1 min after irradiation with UV and continuously elevates up to 10 min, followed by being undetectable 30 min after far-ultraviolet C (UV, principally 254nm wavelength) irradiation in human cells (2-4). This time course is quite different from that in other previously reported proteases requiring more than several hours after UV irradiation (5-14). On the other hand, protease induction promptly after UV irradiation is associated with cellular hypomutable change (2-4, 15-18). Therefore, it is highly important to characterize the UV-induced proteases.

We have reported here partial purification of the UV-induced proteases and induction of other proteases in X-ray-irradiated human cells.

MATERIALS AND METHODS
1. Cells and culture conditions
The human cell lines, UVAP-2 and RSAa, were described elsewhere (12, 19, 20). The medium for cell culture was Eagle's minimal essential medium (EMEM) containing 10% (V/V) calf serum and antibiotics (100 μg/ml streptomycin and 100 units/ml penicillin G). Cells were cultured with the medium at 37°C in a humidified atmosphere containing 5.0% CO2.

2. UV and X-ray irradiation
Conditions for UV and X-ray irradiation of cells were described elsewhere (21, 22).

3. Preparation of protease samples
Protease preparation was performed principally according to the method described previously (23). Cells were irradiated with UV or mock irradiated, following by culture with EMEM. After culturing for 10 min, the EMEM was removed and 1.0 ml of cold lysis buffer was then added to each dish. The lysis buffer consisted of 50 mM HEPES-NaOH (pH 7.5), 1.0 mM EDTA, 0.1% octanoyl-N-methylglucamide (MEGA-8) and 5.0 μM chloroquine. Cell lysates were centrifuged, and the supernatant was used as the protease preparation. The preparation was applied to column chromatography.

4. Assay of protease activity
Levels of fibinolytic protease activity were estimated as described elsewhere (15). Briefly, the protease samples were incubated in 125I-fibrin-coated tubes at 37°C for 15 min. The radioactivity (cpm) of 125I released was counted. The radioactivity increased linearly during incubation for 1 h. Reactions were performed in duplicate. Results were expressed as the total amount of radioactivity per 1.0 x 10^5 cells, after subtracting the radioactivity of control samples from mock-treated cells.

5. Assay of K-ras codon 12 mutation
Base substitution mutation at codon 12 of K-ras was also examined according to the PCR and differential dot-blot hybridization methods, as described previously (24, 25). Briefly, genomic DNA from cells was amplified by PCR using the primers for the codon 12 of K-ras (5'-GACTGAATATAAAGGTGTCG-3' and 3'-GCTTATACGAGCCGTAGG-5'). The amplified DNA was dot-blotted onto nylon membranes. After hybridization with digoxigenin-11-dUTP-3'-end–labeled K-ras codon 12 probes, the membranes were washed and blocked with a blocking reagent. They were then incubated with polyclonal sheep anti-Dig Fab conjugated to alkaline phosphatase (Boehringer Mannheim, Germany) and colored with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate solution (Boehringer Ingelheim, Germany). As a normal probe, the oligonucleotide 5'-GTTGGAGCTGTGGCGTAGG-3' was used, whereas mutant probes contained the following oligonucleotides mixed at the same concentration ratios:
5'-GTTGGAGCTGTGGCGTAGG-3', 5'-GTTGGAGCTGTGGCGTAGG-3', 5'-GTTGGAGCTGTGGCGTAGG-3', 5'-GTTGGAGCTGTGGCGTAGG-3', 5'-GTTGGAGCTGTGGCGTAGG-3', 5'-GTTGGAGCTGTGGCGTAGG-3', 5'-GTTGGAGCTGTGGCGTAGG-3', 5'-GTTGGAGCTGTGGCGTAGG-3',
5'-GTTGGAGCTGCTGGCGTAGG-3' and 5'-GTTGGAGCTGTTGGCGTAGG-3'.

6. Other conditions
All experiments were performed under dim light or a yellow lamp (National, FL 20S-Y-F; Matsushita Electric Industrial Co., Osaka, Japan).

RESULTS
1. Purification of UV-induced proteases
One of the UV-induced proteases from UVAP-2 cells was purified by column chromatography, as shown in Table1. The fraction showing the induced protease activity on 2nd TSK-gel G2000SW contained approximately 30 k proteins. The protease was about 30-fold purified by the gel chromatography (Table1).

Table 1. Purification of a UV-induced protease.

<table>
<thead>
<tr>
<th></th>
<th>Total activity (cpm x 10^3)</th>
<th>Total protein (mg)</th>
<th>Specific activity (cpm/mg protein)</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell lysates</td>
<td>7860</td>
<td>180</td>
<td>44</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>DEAE-Sepharose CL-6B</td>
<td>525</td>
<td>17.5</td>
<td>30</td>
<td>0.68</td>
<td>6.7</td>
</tr>
<tr>
<td>TSK-gel G2000SW</td>
<td>240</td>
<td>7.5</td>
<td>32</td>
<td>0.73</td>
<td>3.1</td>
</tr>
<tr>
<td>Benzamidine-Sepharose 4B</td>
<td>80</td>
<td>0.30</td>
<td>267</td>
<td>6.1</td>
<td>1.0</td>
</tr>
<tr>
<td>Mono Q</td>
<td>38</td>
<td>0.035</td>
<td>1090</td>
<td>25</td>
<td>0.48</td>
</tr>
<tr>
<td>2nd TSK-gel G2000SW</td>
<td>13</td>
<td>0.010</td>
<td>1300</td>
<td>30</td>
<td>0.17</td>
</tr>
</tbody>
</table>

2. Inhibitor sensitivity of UV-induced proteases
Inhibitory effects of protease inhibitors were examined in the protease activity in the enzymatic peak fractions. Antipain inhibited the activity to a great extent, while leupeptin and elastatinal did not (data not shown).

3. X-ray sensitivity of RSa cells
As previously reported (22), RSa cells are susceptible to X-ray cell-killing and show 1.2 Gy of D_0 value by colony-formation analysis. To elucidate X-ray-affected cellular events of RSa cells, we assessed fibrinolytic protease activity in cell extracts. The activity was elevated very early after X-ray irradiation, being detected 10min-30min after irradiation reaching a peak at 10 min (Fig.1A). After the peak, the protease activity level decreased and the elevation disappeared 60 min after X-ray irradiation (Fig.1A). Of the X-ray doses tested (0 Gy-4Gy) 30 min after irradiation the elevation of protease activity was greatest at 2 Gy (Fig. 1B).

Sensitivity of the induced proteases to various protease inhibitors was also tested and leupeptin inhibited the activity to a greater extent than did antipain and elastatinal (data not shown).
Figure 1. Induction of protease activity in RSa cells mock-irradiated and irradiated by X-ray at various times (A) and doses (B).

4. Mutability of RSa cells after X-ray irradiation

The human cell line RSa exhibits hypermutability after UV irradiation and is, therefore, useful in investigating molecular mechanisms of mutation events. It is very easy for us to detect induction of base substitution mutation of K-ras codon 12 in the genomic DNA of UV-irradiated RSa cells, assessed by PCR and differential dot-blot hybridization (25). Therefore, X-ray-induced mutagenecity in RSa cells was tested by the analysis of mutations at K-ras codon 12. Hybridization signals with mutant probes were detected in cells after 6 days of 1 Gy X-ray irradiation (Fig. 2). However, signals with mutant probes were not detected in cells irradiated with higher doses, up to 4 Gy (Fig. 2).

Figure 2. Differential dot-blot hybridization analysis of K-ras codon 12 in X-ray-irradiated and mock-irradiated RSa cells.

DISCUSSION

The present study succeeded in characterization of proteases induced immediately after UV irradiation in human cells. This success was due to utilization in human cells and to utilization of UVAP-2 cells which have
high levels of antipain-sensitive protease activity after UV irradiation. The present study furthermore revealed protease activity induced early after X-ray irradiation in X-ray sensitive human RSa cells.

Serine, and the cysteine type protease inhibitor antipain, suppressed protease activity induced by UV and showed little inhibitory effects on X-ray-induced protease activity. The discrepancy of protease inhibitor susceptibility between UV- and X-ray-induced proteases may imply different molecules of the protease or existence of different active sites of one kind of protease molecules.

We previously found that antipain sensitive protease induction is associated with increased resistance of human cells to UV cell-killing and mutagenicity, as summarized in Table 2. Therefore, we have proposed that the proteases may play a role in UV refractoriness of human cells. Interestingly, RSa cells with protease inducibility after X-ray irradiation showed detectable K-ras codon 12 mutations after 1 Gy X-ray. Thus, X-ray-induced proteases in RSa cells might play a role in X-ray-susceptibility. Efforts are underway to purify UV- and X-ray-induced proteases. The results of this investigation provide the basis for further analysis of the biological function of these proteases in human cells.

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REFERENCES


