A dietary carcinogen, 3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole (Trp-P-1) at 20 μM activates caspase-3-like proteases as an apoptotic marker in rat splenocytes. The present study demonstrated 100 μM Trp-P-1 induced necrosis with activation of caspase-3-like proteases. The activation in necrosis and apoptosis resulted from the activation of caspase-9 and caspase-8, respectively. Thus, Trp-P-1 induces apoptosis and necrosis with the activation of different caspases.

Key words: 3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole (Trp-P-1); apoptosis; necrosis; caspases; splenocyte

3-Amino-1,4-dimethyl-5H-pyrido[4,3-b]indole (Trp-P-1) is a tryptophan pyrolysate produced during the cooking of meat and fish. Previous studies have shown that Trp-P-1 induces apoptosis in rat and human immunocytes and rat hepatocytes, and that Trp-P-1-induced cell death might be associated with various diseases including immunodeficiency. Apoptosis is a distinct form of cell death from necrosis accompanied by characteristic events including DNA fragmentation and morphological changes. Our previous study showed that DNA fragmentation in splenocytes is induced by Trp-P-1 at less than 20 μM in a dose-dependent manner, but is attenuated by a higher concentration of Trp-P-1. Thus, the higher concentration of Trp-P-1 may induce necrosis. Certain chemicals induce both apoptosis and necrosis depending on concentration. For example, 6-hydroxydopamine induces apoptosis at 25 μM and necrosis at 50 μM in PC12 cells, and diamide induces apoptosis at 200 μM and necrosis at 500 μM in Jurkat T cells. These reports indicate an activation of caspase-3-like proteases in apoptosis but not in necrosis. Activation of caspase-3-like proteases is accepted as an apoptotic marker, although a few reports indicate activation of caspase-3-like proteases in non-apoptotic cells such as mitogen-stimulated T cells.

In the present study, to elucidate changes in the activity of caspases in Trp-P-1-caused cell death, rat splenocytes were treated with various concentrations of Trp-P-1 (Wako Pure Chemical Industries, Osaka, Japan), which is dissolved in dimethyl sulfoxide (DMSO) at 40 mM as the stock solution. All animal treatments in this study conformed to the “Guidelines for the care and use of experimental animals, in Rokkodai Campus, Kobe University.” Splenocytes were isolated from male Wistar rats (7 to 8 weeks old, purchased from Japan SLC, Shizuoka, Japan) as described previously. Cells were cultured at a density of 5 × 10^6 cells/ml in RPMI 1640 medium (Nissui Pharmaceutical, Tokyo, Japan) containing 5% fetal bovine serum (Dainippon Pharmaceutical, Tokyo, Japan), 1 mM glutamine and 1 mg/ml kanamycin under an atmosphere of 95% air and 5% CO_2 at 37°C.

The effects of Trp-P-1 on cell viability were determined as total mitochondrial dehydrogenase activity in viable cells using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, with a slight modification as described previously. Trp-P-1 decreased cell viability. The time for a 50% decrease in cell viability (t_{1/2}) of 100 μM Trp-P-1 was almost 30 min, while that of 20 μM was 3 h (Fig. 1A). Morphological changes were observed by phase-contrast microscopy 4 h after treatment with Trp-P-1 (Fig. 1B). In comparison with control cells (Fig. 1B, upper panel), 20 μM Trp-P-1 caused cell shrinkage (Fig. 1B, middle panel), and 100 μM caused cell swelling (Fig. 1B, lower panel).
These changes are characteristic of apoptosis and necrosis, respectively. As a biochemical marker for apoptosis, DNA fragmentation was determined by quantitative measurement using 4',6-diamino-2-phenylindole (DAPI), a fluorescence probe for double-stranded DNA, and by detection of DNA ladder structure using methods reported previously. Trp-P-1 at 20 μM increased DNA fragmentation (Fig. 1C) and DNA ladder structure (Fig. 1D) time-dependently, while 100 μM Trp-P-1 decreased DNA fragmentation (Fig. 1C). These results clearly suggest that 100 μM Trp-P-1 induced necrosis in rat splenocytes, whereas 20 μM Trp-P-1 induced apoptosis consistently with our previous report. We also observed necrotic cell death, which was estimated by both biochemical and morphological markers, in 40 μM Trp-P-1-treated cells.

To investigate the involvement of caspases in Trp-P-1-induced apoptosis and necrosis, the activity of caspases was measured using fluorogenic peptide substrates (Peptide Institute, Osaka, Japan): Acetyl-Asp-Glu-Val-Asp-methylcoumarylamide (Ac-DEVDF-MCA), Ac-Ile-Glu-Thr-Asp-MCA (Ac-IETD-MCA), and Ac-Leu-Glu-His-Asp-MCA (Ac-LEHD-MCA), respectively. The values are presented as % of the activity of control cells treated with DMSO at each time point, and data are expressed as means ± S.D. of triplicate experiments.
Trp-P-1 Increases Intracellular Levels of ROS. (Fig. 2A). Furthermore, the activation was detected 60 min after treatment with 100 \( \mu M \) Trp-P-1, and was earlier than that with 20 \( \mu M \), Trp-P-1 at 20 \( \mu M \) activated caspase-8 at 60 min (Fig. 2B) and caspase-9 at 120 min (Fig. 2C), indicating that 20 \( \mu M \) Trp-P-1 activated a caspase-8-dominated apoptotic pathway. These results are consistent with Trp-P-1-induced apoptosis in mononuclear cells.\(^3\) On the other hand, 100 \( \mu M \) Trp-P-1 activated caspase-9 at 60 min (Fig. 2C) without activating caspase-8 (Fig. 2B), indicating that 100 \( \mu M \) Trp-P-1 activated caspase-3-like proteases following the activation of caspase-9 during necrosis. Thus, activation of caspase-9 and caspase-3-like proteases is unlikely to constitute firm general evidence for apoptosis, although these proteases have been accepted as apoptotic markers.

It has been reported that caspase-9 is activated by reactive oxygen species (ROS) in a mitochondrial pathway of apoptosis.\(^{12}\) Therefore, the involvement of ROS in Trp-P-1-induced cell death was investigated by a spectrofluorometrical method\(^3\) using 2',7'-dichlorofluorescein diacetate (DCFH-DA), a cell-permeable probe for ROS. When splenocytes were incubated for 1 h with Trp-P-1, intracellular ROS increased in a dose-dependent manner (Fig. 3). This result is consistent with previous reports. Trp-P-1 produces ROS in rat mononuclear cells from blood in a dose-dependent manner,\(^2\) and intraperitoneally administered Trp-P-1 increases thiobarbituric acid reactive substances in the liver of rats.\(^3\) ROS produced in small quantities by 20 \( \mu M \) Trp-P-1 (Fig. 3) might not be associated with apoptosis, because caspase-8-mediated apoptosis is believed to be dependent on death receptors but not on ROS.\(^8\) Hydrogen peroxide at more than 50 \( \mu M \) induces necrosis in Jurkat T cells.\(^{14}\) Free radical generators, Fe\(^2+\) (10 \( \mu M \)) and L-buthionine-[\( \Sigma \),\( \delta \)]-sulfoximine (1 mM) induce necrosis in rat striatal cell culture.\(^{15}\) The high amount of ROS produced by 100 \( \mu M \) Trp-P-1 might be associated with necrosis. Thus, Trp-P-1-produced ROS may contribute to Trp-P-1-activated caspase-9 during necrosis, but not to Trp-P-1-activated caspase-8 during apoptosis. However, caspase-9 is unlikely to initiate necrosis, because \( t_{1/2} \) of 100 \( \mu M \) Trp-P-1 was 30 min (Fig. 1A), whereas caspase-9 was not activated at 30 min (Fig. 2C). Trp-P-1-produced ROS probably activated caspase-9 in the case of necrosis, although it is unclear whether ROS is the cause or the result of necrosis. Further study is needed to understand the relationship between ROS generation and caspase activation.

References

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