Protective Effect of Tetrandrine on Normal Human Mononuclear Cells against Ionizing Irradiation

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Tetrandrine, an alkaloid isolated from the plant *Stephania tetrandra*, at low concentration (2 $\mu\text{g/ml}$) was shown to protect normal human mononuclear cells *in vitro* against damage due to a single high-dose of ionizing irradiation (10 Gy). The cell survival rate increased from $58.3 \pm 2.2\%$ in the irradiated group to $78.0 \pm 2.6\%$ in the tetrandrine-pretreated group, and similarly, the percentage of necrotic cells declined from $20.7 \pm 2.5\%$ to $10.7 \pm 1.9\%$, respectively. This protective effect of tetrandrine for cell surviving fraction increased in a dose-dependent manner. Tetrandrine was also found to inhibit inflammatory responses induced by irradiation including the release of superoxide (NBT [nitroblue tetrazolium] reduction decreased from $21.3 \pm 2.3\%$ to $10.2 \pm 2.5\%$) and phagocytic activity (decreased from $80.7 \pm 3.8\%$ to $50.7 \pm 2.3\%$, the same range level as that of the control group). However, the alkaloid demonstrated no effect on the production of nitric oxide. In terms of cell morphology, only two types were observed—normal or necrotic cells, and there were no characteristics of programmed cell death. These results indicate that tetrandrine possesses radioprotective activity against 10 Gy of ionizing irradiation and could suppress irradiation-induced inflammatory processes.

Key words tetrandrine; radioprotective activity; mononuclear cell

Tetrandrine [(1 β)-6,6',7,12-teramethoxy-2,2'-dimethylberbaman] is a bisbenzylisoquinoline alkaloid¹⁾ isolated from the dried root of *Stephania tetrandra* S. MOORE. Its empirical formula is $\text{C}_{38}\text{H}_{42}\text{O}_6\text{N}_2$, molecular weight 622.73 and its structure is composed mainly of two 17C rings connected by a double oxygen bridge. The compound is practically insoluble in water, and according to accounts in the literature, exerts a variety of pharmacological effects as follows:

Firstly, the action of anti-inflammation is achieved by inhibiting the production of inflammatory mediators (TNF- α , TNF- β , IL-1 β , etc.) and intermediates of inflammation (e.g., prostaglandin, leukotriene, nitric oxide)^{2,3)} by monocytes, macrophages and lymphocytes (collectively termed mononuclear cells). Secondly, tetrandrine can achieve the effect of anti-hypertension by relaxing arterial smooth muscles directly and decreasing the average arterial pressure.⁴⁾ Thirdly, it acts as a Ca^{2+} channel blocker including blocking action against voltage-dependent L-type and T-type Ca^{2+} channels.^{5,6)} There are also other effects achieved by this compound such as anti-arrhythmia,⁷⁾ anti-ischemia⁸⁾ and anti-fibrosis of the lung.⁹⁾

Radiation therapy (RT) employing ionizing irradiation can kill tumor cells, but also causes significant damage to normal tissues and cells, of which the severely acute and subacute inflammatory reactions may be fatal. In the literature, there were some investigations showing that natural alkaloids play a role in radioprotective activity. For example, vincanor (isolated from Vinca alkaloids) protects rats against lethal gamma-radiation,¹⁰⁾ and the alkaloid fraction of *Panax ginseng* reduces lymphocyte damage caused by gamma-rays.¹¹⁾ The purpose of this study was to determine whether tetrandrine, a natural alkaloid, could reduce the damage sustained by mononuclear cells when subjected to a single high-dose of

ionizing irradiation. In addition to counting the change in cell viability in each test group, the activities of superoxide release, phagocytosis and nitric oxide production were also assayed.

MATERIALS AND METHODS

Preparation of Tetrandrine Solution Dried powder purchased from Aldrich Co. (Milwaukee, U.S.A.), 98% in purity, was dissolved in 1 M HCl and the pH of the solution was then adjusted to 7.0 with 5 M NaOH. The resulting solution was divided into aliquots (20 mg/ml). At the time of testing, aliquots were diluted with Roswell Park Memorial Institute (RPMI) 1640 medium (GIBCO, Grand Island, U.S.A.) and cell fluid added to obtain a solution with a concentration of 2 $\mu\text{g/ml}$. The mononuclear cells were incubated with the tetrandrine solution for 30 min before being subjected to irradiation.

Isolation of Mononuclear Cells Under sterile operating conditions, 50 ml of whole blood from healthy young volunteers was drawn into an empty syringe preloaded with 1 ml heparin (5000 units/ml, Leo Pharmaceutical Products, Denmark) and the blood was divided into test tubes prefilled with 5 ml Ficoll-Hypaque (1.077 gm/ml, Pharmacia Fine Chemicals, Uppsala, Sweden). The tubes were centrifuged at $400 \times g$ for 30 min followed by subsequent extraction of the white interphase in the middle. The extract was placed in a test tube containing RPMI 1640 medium and centrifuged at $200 \times g$ for 10 min. The cells were then washed twice with RPMI 1640, the supernatant was removed and solution containing RPMI 1640 and 10% heat-inactivated fetal calf serum (FCS) (Hyclone, Logan, U.S.A.) was added to produce the mononuclear cell suspension. The number of cells was counted with a counting chamber under an upright microscope and the cell concentration was adjusted to

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1.5×10^6 /ml. Five ml aliquots of each of the resulting suspensions were incubated after the treatment of various drugs. The incubation conditions were 37°C and 5% CO_2 .

Test Groups The cell preparations were divided into five groups: the control group received no treatment; the phytohaemagglutinin (PHA, Sigma, St. Louis, U.S.A.) group was treated with $5 \mu\text{g}/\text{ml}$ PHA for 30 min; the irradiated group was subjected to a single dose of 10 Gy irradiation; the tetrandrine group was treated with $2 \mu\text{g}/\text{ml}$ tetrandrine for 30 min; and the tetrandrine plus irradiation group was treated with $2 \mu\text{g}/\text{ml}$ tetrandrine for 30 min followed by 10 Gy irradiation.

Ionizing Irradiation Treatment Mononuclear cells in the irradiated groups were subjected to a single dose of 10 Gy irradiation simultaneously using a Cobalt-60 apparatus (Theratron T780C, Canada, dose rate $1.6 \text{ Gy}/\text{min}$). The drugs were removed from each treated group by washing 30 min after irradiation and the cell concentrations were adjusted to 1.5×10^6 /ml. Then, each group was incubated for 24 h.

Viability Assay The drugs in the test groups were removed 30 min after irradiation, then the baseline numbers of viable and dead cells were counted using the trypan blue (GIBCO, Grand Island, U.S.A.) exclusion test, and the cell concentrations were adjusted to 1.5×10^6 /ml. Cell counts were performed again after 24 h of incubation to determine the changes in viability, and the surviving fraction and death ratio were calculated by the following equations:

- (i) Surviving fraction =
$$\frac{\text{Number of viable cells in each group after 24 h}}{\text{Number of viable cells in control group after 24 h}}$$
- (ii) Death ratio =
$$\frac{\text{Number of dead cells in each group after 24 h}}{\text{Number of dead cells + Number of viable cells in the same group}}$$

NBT Reduction Test A large amount of superoxide is produced by monocytes when stimulated by phorbol myristate acetate (PMA) which can reduce the water-soluble yellow-colored nitroblue tetrazolium (NBT) (Sigma, St. Louis, U.S.A.) to a blue-black formazan precipitate. The test was developed by Baehner and Nathan¹²⁾ and modified as follows: $0.5 \text{ ml } 1 \times 10^6$ /ml mononuclear cells were pipetted into a test tube and 0.5 ml NBT solution (containing NBT $2 \text{ mg}/\text{ml}$ and $1 \mu\text{M}$ PMA) added. The test tube was incubated for 30 min, and then $80 \mu\text{l}$ of the mixture was taken out and cytocentrifuged onto a slide using a Cytospin^{2R} (Shandon Southern Instrument Inc., Germany). The slide was dried and stained with Safranin O (Sigma, St. Louis, U.S.A.) for contrast staining. Two hundred cells were observed under a microscope and the percentage containing formazan was counted.

Phagocytic Activity Assay This method was designed by Gallagher *et al.*¹³⁾ and modified as follows: yeast suspension and mononuclear cell suspension were mixed in a $12 \times 75 \text{ mm}$ tube to produce $100 \mu\text{l}$ mixture with the ratio of yeast to mononuclear cells at 4:1 (4×10^6 /ml: 1×10^6 /ml). The resulting mixture was incubated for 30 min and placed on ice. Fifteen μl of this solution was

pipetted onto a slide, 200 cells were observed under a microscope and the percentage which had ingested yeast was counted.

Observation of Cell Morphology Fifty μl of each of the 1×10^6 /ml cell solutions was removed and cytocentrifuged onto slides using a Cytospin^{2R}. The cell morphology was observed following Wright's staining.¹⁴⁾

Determination of Nitric Oxide Nitric oxide production by mononuclear cells was measured by the amount of nitrite detected in the culture medium using spectrophotometry based on the Griess reaction.¹⁵⁾ One hundred μl of each of the 1.5×10^6 /ml cell solutions was placed in a 24-well plate and incubated for 24 h after the addition of $10 \mu\text{g}/\text{ml}$ lipopolysaccharide and $50 \text{ U}/\text{ml}$ IFN- γ . After incubation, $100 \mu\text{l}$ of supernatant was added to each well with an equal volume of Griess reagent (containing 1% sulfanilamide, 0.1% *N*-1-naphthylethylenediamine and 3% H_3PO_4). The plate was left undisturbed at room temperature for 10 min and the absorbance measured at a wavelength of 550 nm using spectrophotometry. The results were calculated based on the standard curves derived from nitrite standards.

Statistic Analysis The significant difference was evaluated by unpaired Student's *t*-test and accepted as *p* value less than 0.05.

RESULTS

Changes in Cell Death Ratio The death ratio was $20.7 \pm 2.5\%$ in the irradiated group and this was significantly decreased to $10.7 \pm 1.9\%$ ($p = 0.03$) in the group treated with irradiation following $2 \mu\text{g}/\text{ml}$ tetrandrine administration. The death ratio of both control and the tetrandrine group was less than 5% (Fig. 1).

Comparison of Cell Viability The surviving fraction of mononuclear cells after 24-h incubation in the irradiated group and irradiation following $2 \mu\text{g}/\text{ml}$ tetrandrine administration was $58.3 \pm 2.0\%$ and $78.0 \pm 2.6\%$ ($p = 0.004$),

Fig. 1. Effects of Various Treatments on Death Ratio of Mononuclear Cells

Mononuclear cells (1.5×10^6 /ml initially) were cultured for 30 min in the absence or presence of tetrandrine ($2 \mu\text{g}/\text{ml}$) with/without following RT (10 Gy). Viable and dead cells were counted after 24-h culture and the death ratio was calculated. Results from three experiments are expressed as means $\pm$ S.E.M. * Significant decrease from RT alone ($p = 0.03$).

Fig. 2. Effects of Various Treatments on Surviving Fraction of Mononuclear Cells

Mononuclear cells (1.5×10^6 /ml initially) were cultured for 30 min in the absence or presence of tetrandrine ($2 \mu\text{g}/\text{ml}$) with/without following RT (10 Gy). Viable cells were counted after 24-h culture and calculated for surviving fraction. Results from three experiments are expressed as means $\pm$ S.E.M. * Significant increase from RT alone ($p=0.004$).

Fig. 3. Effects of Various Concentrations of Tetrandrine on Surviving Fraction of Mononuclear Cells with Ionizing Irradiation

Mononuclear cells (1.5×10^6 /ml initially) were cultured for 30 min in the presence of serially diluted tetrandrine (0– $4 \mu\text{g}/\text{ml}$) with following RT (10 Gy). Viable cells were counted after 24-h culture and the surviving fraction was calculated. Results from three experiments are expressed as means $\pm$ S.E.M.

respectively (Fig. 2). This protective effect against RT increased with increasing dose of tetrandrine (with surviving fraction of $58.3 \pm 2.2\%$ for $0 \mu\text{g}/\text{ml}$, $58.8 \pm 1.9\%$ for $0.5 \mu\text{g}/\text{ml}$, $65.2 \pm 2.3\%$ for $1 \mu\text{g}/\text{ml}$, $78.0 \pm 2.6\%$ for $2 \mu\text{g}/\text{ml}$ and $83.1 \pm 2.3\%$ for $4 \mu\text{g}/\text{ml}$, respectively.) (Fig. 3). The same surviving fraction in the control group was $106.3 \pm 3.6\%$ and was $81.2 \pm 3.0\%$ in the group treated with tetrandrine alone.

NBT Reduction Reaction After 24 h of incubation, only $4.3 \pm 0.9\%$ of monocytes in the control group, $21.3 \pm 2.3\%$ in the irradiated group and $10.2 \pm 2.5\%$ in the tetrandrine plus irradiation group exhibited a positive NBT reduction reaction (Fig. 4). The positive NBT

Fig. 4. NBT-Reducing Activity of Mononuclear Cells after the Same Treatments as Described in Legend to Fig. 1

The harvested cells were assayed for NBT-reducing activity and the percentage of positively stained cells among 200 cells was counted under a microscope. Results from three experiments are expressed as mean percentages $\pm$ S.E.M. * No significant difference ($p=0.85$). ** Significant decrease from RT alone ($p=0.03$).

Fig. 5. Phagocytic Activity of Mononuclear Cells after the Same Treatments as Described in Legend to Fig. 1

The harvested cells were assayed for phagocytic activity and the percentage of yeast-ingested cells among 200 cells was counted under a microscope. Results from three experiments are expressed as mean percentages $\pm$ S.E.M. * No significant difference ($p=0.90$). ** Significant decrease from RT alone ($p=0.007$).

reduction rate of the latter group was greater than that of the control group, but markedly lower than that of the irradiated group ($p=0.03$). In addition, the positive NBT reduction rate in the tetrandrine group was $4.6 \pm 1.5\%$ which was not significantly different from that of the control group ($p=0.85$).

Phagocytic Activity After 24 h of incubation, $50.7 \pm 2.3\%$ of monocytes in the control group exhibited phagocytic activity for yeast. In comparison, $80.7 \pm 3.8\%$ of monocytes in the irradiated group and $51.3 \pm 4.3\%$ in the tetrandrine plus irradiation group showed phagocytic activity for yeast (Fig. 5). The phagocytic activity of the latter group was thus significantly different from that of the irradiated group ($p=0.007$) but not statistically different from that of the control group ($p=0.90$). In addition, $52.1 \pm 3.1\%$ of monocytes in the tetrandrine group exhibited phagocytic activity which was also not significantly different from that of the control group. In

the PHA group, $60.5 \pm 3.7\%$ of cells displayed phagocytic activity.

Nitric Oxide Production The concentration of nitric oxide produced by cells in each group did not vary markedly, with a range of $0.1\text{--}1.5\ \mu\text{M}$.

Cell Morphology Either viable or necrotic mononuclear cells were observed under light microscope. There were no characteristics of apoptosis seen in any preparations of stained cells.

DISCUSSION

The results indicate that a single dose of 10 Gy irradiation can kill 40% of mononuclear cells, whereas the same dose kills only 20% of cells if they are pretreated with $2\ \mu\text{g/ml}$ tetrandrine. Although tetrandrine showed a low level of inhibitory activity toward cell viability, the dead cell ratio in the same group was not higher than that of the control group, suggesting that tetrandrine only inhibits cell proliferation, but does not destroy normal mononuclear cells. The report by Seow *et al.* in 1992 also confirmed that even $20\ \mu\text{g/ml}$ tetrandrine is not toxic to mononuclear cells and neutrophils.²⁾ There was no such protective effect when tetrandrine was given after irradiation (data not shown) in a preliminary study.

In terms of the physiological functions of the mononuclear cells, NBT reduction is usually employed to test the myeloid cell activity in superoxide production.¹⁶⁾ Of the mononuclear cells, monocytes belong to this type of cell and the release of superoxide is closely associated with their bactericidal activity and inflammatory response. In this study, only 4.3% of monocytes in the control group gave a positive NBT reduction reaction. This proportion was elevated to 21.3% in the irradiated group but reduced to 10.2% in the tetrandrine plus irradiation group, suggesting that tetrandrine may inhibit the superoxide production of irradiation-stimulated monocytes. Whether this inhibition goes through direct reaction with O_2^- generation or superoxide dismutase (SOD) activity remains unclear. However, the inhibitory effect of tetrandrine on neutrophil function *via* increase in both SOD activity and cAMP level has been reported.¹⁷⁾ On the other hand, in the phagocytic activity experiment, 50.7% of cells in the control group exhibited such activity with the proportion increased to 80.7% in the irradiated group; that of the tetrandrine plus irradiation group was reduced to the level of the control group, demonstrating that tetrandrine can markedly inhibit the phagocytic activity of irradiation-activated monocytes. The responses of irradiation-activated monocytes were similar to the inflammatory reactions induced in bacterial infection. According to the aforesaid tests of NBT reduction and phagocytic activity, such inflammatory-like activation could be partially (NBT reduction) or completely (phagocytic activity) inhibited by tetrandrine. These results were similar to the activity of tetrandrine in inhibiting the release of inflammatory mediators induced by *Staphylococcus aureus*.²⁾ Thus, we assume that tetrandrine could inhibit the inflammatory processes of mononuclear cells induced by irradiation and this inhibition is closely related to its protective activity against injury by irradiation. In fact,

assays for the gene expression of early-stage inflammatory mediators induced by irradiation, such as TNF- α mRNA, TNF- β mRNA, IL-6 mRNA and IL-1 mRNA will constitute one direction of our future studies.

Accounts in the literature have shown that ionizing irradiation may induce some normal¹⁸⁾ and tumor cells^{19,20)} to initiate programmed death. However, in this study normal cells subjected to a single high dose of irradiation did not produce the morphological characteristics of programmed death after 24 h, and only two morphological types were observed—those of normal and necrotic cells. In view of the fact that morphological changes occur later than DNA changes, programmed death (or the lack of it) needs to be verified by the presence of DNA fragmentation under electrophoresis or the distribution of Sub-G1 phase in the cell cycle analysis under flow cytometry.

In the nitric oxide test our results were similar to those presented in many publications²¹⁾ and indicated that nitric oxide production by human monocytes in each of the test groups was not affected by any of the treatments aforementioned, being in the base range of $0.1\text{--}1.5\ \mu\text{M}$.

Because the importance of physical interactions between lymphocytes and monocytes was previously emphasized in cytokine-induced NO production,²²⁾ we used peripheral mononuclear cells including both cells instead of pure monocytes for assay of the inflammatory process in this study.

Some known calcium channel blockers such as diatiazem (a benzothiazapine), which are used widely in cardiovascular treatment, also possess a protective effect against death by ionizing irradiation in mice.²³⁾ Their radioprotection may be due to interference with the damaging cellular influx of Ca^{2+} after radiation-induced cell membrane injury.²³⁾ Being one kind of Ca^{2+} channel blocker, tetrandrine may also protect mononuclear cells against irradiation *via* a similar mechanism. However, while considering the diverse pharmacological effects of Ca^{2+} channel blockers, further investigation focusing on the correlation between Ca^{2+} channel blocking activity and radioprotective effect is needed to confirm the above assumption.

The main structural characteristics of tetrandrine include (1) methoxy groups at C_7 and C_{12} ; (2) uncharged nitrogens at N_2 and N'_2 ; and (3) a double oxygen-bridged ring of 18 bond lengths.²⁴⁾ Some studies comparing the pharmacological activities between tetrandrine and berbamine (which differs from tetrandrine only in the methoxyl substitution of the hydroxyl group at the 12-C position in one benzyl ring) indicated that the methoxyl group in the benzyl ring is crucial for both Ca^{2+} channel blockade and inhibition of production of inflammatory cytokines by mononuclear cells.^{2,25)} In this connection, the radioprotective activity of tetrandrine may be provided by the above functional group and further study of its structure-activity relationship is worthwhile.

In conclusion, a low dose of tetrandrine partially protected mononuclear cell viability from a high dose of ionizing irradiation associated with suppression of irradiation-induced inflammatory processes.

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