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INDUCTION OF RESISTANCE TO TNF CYTOTOXICITY AND MITOCHONDRIAL SUPEROXIDE DISMUTASE ON U-937 CELLS BY 1,25-DIHYDROXYVITAMIN D_3

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SUMMARY: 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) dose-dependently inhibited the cytotoxicity of tumor necrosis factor (TNF) in a human monoblastic leukemic cell line, U-937. Combination of TNF and $1,25(OH)_2D_3$ remarkably increased mitochondrial superoxide dismutase (mSOD) of U-937 cells, TNF alone increased it only slightly and $1,25(OH)_2D_3$ alone did not. The cytosolic SOD (cSOD) activity was not changed by TNF or/and $1,25(OH)_2D_3$. The mSOD activity was not inhibited by 2 mM KCN, suggesting that mSOD should be a manganase SOD (MnSOD). These results suggest that $1,25(OH)_2D_3$ may reduce the susceptibility to TNF cytotoxicity of U-937 cells by enhancing the ability of inducing MnSOD by TNF.

TNF is a protein originally isolated by its ability to induce hemorragic necrosis of some tumors in vivo (1), and cytotoxic effects against certain tumor cells, but not against normal cells in vitro (2). Many investigators have suggested that normal cells and some resistant cells have certain proteins involved in protection against the cytotoxicity of TNF, since normal cells or resistant cells are killed by TNF when combined with an inhibitor of protein or RNA synthesis (3). Though mechanisms of the cytotoxicity of TNF are not clear, there is much evidence that free radicals such as superoxide and

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ABBREVIATIONS: $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; SOD, superoxide dismutase; TNF, tumor necrosis factor.

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hydroxyl radicals, mediated by TNF, are correlated with TNF cytotoxicity (4, 5). Recently, Wong et al. described that manganase containing SOD (MnSOD) was a rescue protein against TNF cytotoxicity in the resistant cells (6) and was induced when the cells were exposed to TNF or interleukin-1 (7).

SODs are enzymes that detoxify superoxide radicals and interfere with production of hydroxyl radicals (8). There are three forms of SODs characterized by their metal requirements: copper-zinc (Cu/ZnSOD), manganese (MnSOD), and iron (FeSOD). Cu/ZnSOD and MnSOD are predominant in the cytoplasm of most eukaryotic cells, and the matrix of mitochondria, respectively (9). Cu/ZnSOD is alwasys synthesized, but MnSOD is induced by oxygen, and superoxide radical generators (10).

A human monoblastic leukemia cell line, U-937, can be induced to differentiate into a mature monocyte/macrophage pathway by $1,25(OH)_2D_3$ (11), and killed by TNF, which can induce differentiation of other some myelogenous leukemia cell lines (12). Thus, a combination of $1,25(OH)_2D_3$ and TNF synergistically affected induction of differentiation of U-937 cells (13). We present here inhibition of TNF cytotoxicity in U-937 cells by $1,25(OH)_2D_3$, and examine whether MnSOD is related to inhibition of TNF cytotoxicity.

MATERIALS AND METHODS

Materials: $1,25(OH)_2D_3$ was purchased from Phillips Dupher, Amsterdam, the Netherlands. Recombinant human TNF (3×10^6 U/mg protein) was a generous gift from Dainippon Pharmaceutical Co., LTD., Osaka Japan. Nitroblue tetrazolium (NBT) and xanthine oxidase (Grade III from buttermilk) were purchased from Sigma Chemical Company, St.Louis, MO, USA.

Cell line and culture: U-937 cells were cultured with RPMI 1640 medium (GIBCO, Grand Island, NY, USA) containing 10% heat inactivated fetal bovine serum (FBS) (Filtron, Pty., Ltd., Victoria, Australia) in a humid atmosphere of 5% CO_2 at 37°C. The cells used for this study were all established to be free of Mycoplasma contamination by evaluation with Mycoplasma test kits (GEN-PROBE Inc., San Diego, CA USA).

Assay of cytotoxic activity: U-937 cells (3×10^5) were cultured for 3 days in 200 µl of culture medium, in 96 well culture

plates with appropriate concentrations of TNF or $1,25(OH)_2D_3$. Viability of the cells was estimated by trypan blue dye exclusion and percent viability was calculated by counting at least 200 cells in triplicate samples.

Isolation of mitochondrial and cytosolic fractions: Mitochondria was isolated by a modification of the procedure of Schnaitman and Greenawalt (14). U-937 cells (1×10^7) in isolation medium (220 mM manitol, 70 mM sucrose, 2 mM HEPES, 0.5 mg/ml bovine serum albumin, pH 7.4 adjusted by KOH) were homogenized with 30 strokes at 1,000 rpm in ice water by Potter-Elvehijem homogenizer. After removal of nuclei and debris by centrifugation at 560 x g for 15 min, the supernatant was centrifuged at 7000 x g for 15 min. The supernatant was collected as the cytosolic fraction and the precipitate was washed twice by resuspension in one-half and one fourth of the original volume of isolation medium. The precipitate was resolved with sample buffer (1% Triton X-100, 0.25 M sucrose and 10 mM Tris HCl, pH 7.4) and used as the mitochondrial fraction.

Assay of superoxide dismutase activity:

SOD activity was presented in acrylamide gel electrophoresis by a modification of the method of Beauchamp (15), and measured by a modification of the method of MacCord and Fridovich (16).

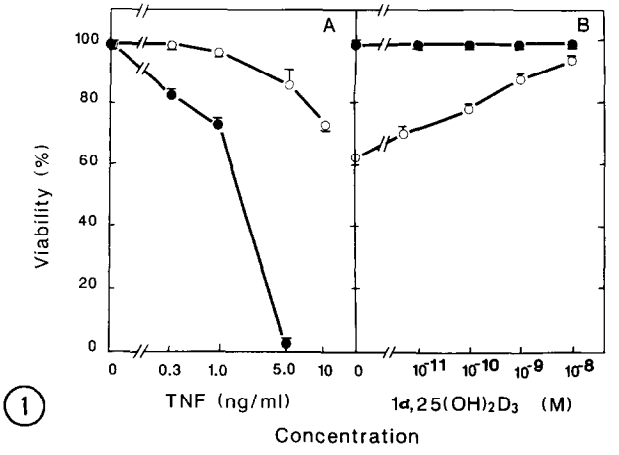
Acrylamide gel assay: An equal quantity of protein measured by Lowry's method modified was applied to the 10% polyacrylamide gel electropholesis. The gel was stained with 2 mg/ml NBT for 15 min in the dark and replaced with 10 μg/ml of riboflavin plus potassium cyanide or riboflavin only in the dark for 15 min. The gel was then illuminated 1 to 3 h. During illumination the gel became unifomly blue except the position containing SOD.

Spectrophotometric assay: Superoxide dismutase was assayed in terms of its ability to inhibit the oxygen-dependent reduction of cytochrome c by xanthine oxidase. The reaction mixture contained 0.1 mM EDTA, 0.1 mM xanthine, 0.01 mM cytochrome c, and 0.05 M potassium phosphate buffer at pH 7.8. The reaction was started by a quantity of xanthine oxidase that produces a rate of cytochrome c reduction at 550 nm of 0.025 absorbance unit per min. Under these difined conditions, the amount of superoxide dismutase required to inhibit the rate of reduction of cytochrom c by 50% (i.e to a rate of 0.0125 absorbance unit per min) was defined as 1 unit of activity and the activity is described as units per mg protein of samples.

RESULTS

Almost all U-937 cells were killed by 5 ng/ml TNF in culture for 3 days. Although, only about 10% of all the cells treated simultaneously with TNF and 10^{-8} M $1,25(OH)_2D_3$ were killed. The treatment with $1,25(OH)_2D_3$ abolished TNF cytotoxic activity to at least less than one tenth that of the control (Fig. 1-A). The inhibition of TNF cytotoxicity by $1,25(OH)_2D_3$ was paralleled with dose of $1,25(OH)_2D_3$ (Fig. 1-B).

We examined SOD activity by two methods: an acrylamide gel assay using NBT dye ; and spectrophotometric assay using



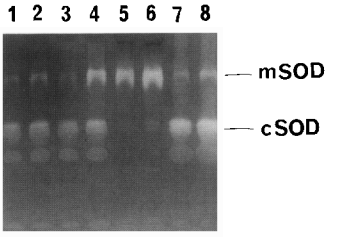


Figure 1. (A) Dose dependent effect of TNF on the viability of U-937 cells in the presence or absence of $1,25(OH)_2D_3$. U-937 cells were cultured for 3 days with TNF at indicated concentrations in the presence (O-O) or absence (●-●) of 10^{-8} M $1,25(OH)_2D_3$. Viability was estimated by trypan blue dye exclusion as described in Materials and Methods. (B) Dose dependent effect of $1,25(OH)_2D_3$ on inhibition of TNF cytotoxic activity against U-937 cells. U-937 cells were cultured for 3 days with $1,25(OH)_2D_3$ at indicated concentrations in the presence (O-O) or absence (●-●) of 2ng/ml TNF. Each point is mean value (n=3) with standard deviation.

Figure 2. SOD activity of U-937 cells treated with TNF or/and $1,25(OH)_2D_3$ in acrylamide gel assay. U-937 cells were cultured for 24 h with none (lane 1), 5 ng/ml of TNF (lane 2), 10^{-8} M of $1,25(OH)_2D_3$ (lane 3), or TNF and $1,25(OH)_2D_3$ (lane 4). Cells (1×10^6) were then lysed by the sample buffer and nuclei and debris removed by centrifugation at 1,000 x g for 5 min. The mitochondrial fraction of the control (lane 5) or cells treated with TNF and $1,25(OH)_2D_3$ (lane 6), and cytosolic fraction of the control (lane 7) or cells treated with TNF and $1,25(OH)_2D_3$ (lane 8) were isolated from 1×10^7 cells. SOD activity of the supernatant was analysed by 10% PAGE as described in Materials and Methods. mSOD and cSOD indicate MnSOD and Cu/ZnSOD, respectively, of main SOD activity in U-937 cells.

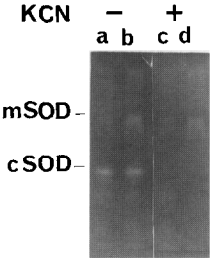
cytochrome c, because cytochrome c oxidase and peroxidase might interfere with the latter assay. Assay of SOD activity was performed 24 hours after exposure of the cells to TNF, because many cells would be killed by treatment with TNF (5 ng/ml) alone during longer exposure. More than 90% of the cells treated were viable.

There were two major bands of SOD activity at higher molecular weight (mSOD) and lower molecular weight (cSOD) (Fig. 2). Almost all cSOD activity was isolated in cytosolic

fraction and disappeared in the presence of 2 mM potassium cyanide (Fig. 3), suggesting that cSOD was Cu/ZnSOD (15). cSOD was not affected by treatment with TNF or/and $1,25(OH)_2D_3$.

Almost all mSOD activity was isolated in mitochondrial fractions and was not reduced in the presence of cyanide, suggesting that mSOD was MnSOD. U-937 cells had little mSOD activity, and this activity was increased greatly by treatment with both TNF and $1,25(OH)_2D_3$, but it was only slightly increased by the tratment with TNF alone and not at all by $1,25(OH)_2D_3$ alone.

We determined SOD activity in those fractions by the spectrophotometric assay (Fig. 4). The activity of mSOD of control was determined to be about 200 U/mg protein. Treatment with both TNF (5 ng/ml) and $1,25(OH)_2D_3$ (10^{-8} M) increased activity to 4-5 times that of the control. The SOD activity of



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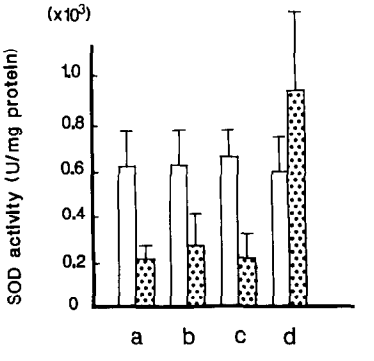

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Figure 3. Inhibition of SOD activity by potassium cyanide. Samples of U-937 cells (1×10^6) treated with (lane b, and d) or without (lane a, and lane c) 5 ng/ml TNF and 10^{-8} M $1,25(OH)_2D_3$ were subjected to 10% PAGE. SOD activity in the gel was analysed in the presence (lane c, and d) or absence (lane a, and b) of 2 mM KCN as described in Materials and Methods.

Figure 4. Effect of TNF or/and $1,25(OH)_2D_3$ on SOD activity in cytosolic or mitochondrial fractions. U-937 cells were cultured with 5 ng/ml TNF or/and 10^{-8} M $1,25(OH)_2D_3$ for 24 h. A fraction of cytosol (□) or mitochondria (◘) was then isolated from 5×10^7 cells and the SOD activity was determined by spectrophotometric assay as described in Materials and Methods. a, control; b, TNF alone; c, $1,25(OH)_2D_3$ alone; d, TNF and $1,25(OH)_2D_3$. Each point is mean value (n=3) with standard deviation.

each cytosolic fraction was the same approximately 600 U/mg protein. These results agreed with the results from acrylamide gel assay.

DISCUSSION

The data presented demonstrate that treatment with 10^{-8} M $1,25(OH)_2D_3$ reduced the susceptibility of U-937 cells to TNF cytotoxic activity to at least less than one tenth that of control and suppression of TNF cytotoxicity by $1,25(OH)_2D_3$ was dose dependent. Since $1,25(OH)_2D_3$ did not changed the number or dissociation constant of TNF receptors on U-937 cells in culture within 24 h (unpublished data), it might modify TNF cytotoxicity at a post receptor level. Then, we examined the activity of SOD, a protein that may protect from TNF cytotoxicity.

mSOD activity was present at approximately one third of cSOD activity in U-937 cells. TNF alone increased mSOD activity only slightly, but its combination with $1,25(OH)_2D_3$ remarkably increased mSOD activity, although cSOD activity was little affected by treatment with TNF or/and $1,25(OH)_2D_3$. As mSOD activity was not inhibited by cyanide, mSOD might be MnSOD. Therefore, induction of MnSOD by $1,25(OH)_2D_3$ might be correlated to resistance to TNF cytotoxicity in U-937 cells, which agrees with Wong *et al.*(6).

Matthews *et al.* reported that the first organelles to appear damaged in TNF-treated cells were mitochondria, and that mitochondrial dysfunction was more significant in the susceptible cell lines than in their resistant sublines (4). Thus, from the point of view that TNF cytotoxicity is mediated by free radicals, the increase of MnSOD activity in mitochondria could explain protection from TNF cytotoxicity.

In our study, $1,25(OH)_2D_3$ enhanced the induction of MnSOD by TNF on U-937 cells although it did not induce MnSOD by itself.

The cells were induced to differentiate and their proliferation was suppressed at the same time (data not shown). It remains to be clarified whether the effect of $1,25(OH)_2D_3$ can affect general tumor cells, or only the cells associated with induction of differentiation. Since $1,25(OH)_2D_3$ at a physiological concentration (10^{-10} M) was effective in inhibiting TNF cytotoxicity of U-937 cells, it may be valuable to clarify the physiological role of $1,25(OH)_2D_3$ in protecting from TNF cytotoxicity and induction of MnSOD.

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