Biochemical Pharmacology, Vol. 60, pp. 1361–1365, 2000.



ISSN 0006-2952/00/$–see front matter
PII S0006-2952(00)00438-X

**SHORT COMMUNICATION**

# Correlation of a Unique 220-kDa Protein with Vitamin D Sensitivity in Glioma Cells

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**ABSTRACT.** We have investigated the antitumor and apoptotic effects of 1,25-dihydroxyvitamin $D_3$ ($VD_3$) in glioma cell lines and in primary cultures derived from surgical specimens from patients. Our results showed that certain glioma cells underwent apoptosis, whereas others were resistant. In an attempt to search for parameters that dictate $VD_3$ sensitivity, we discovered a unique 220-kDa protein in glioma cells that were sensitive to $VD_3$. This protein was not a classical vitamin D receptor (VDR), but was recognized by two different anti-VDR monoclonal antibodies. Furthermore, the level of the 220-kDa protein was inversely correlated with the $IC_{50}$ of $VD_3$ in these glioma cells. This 220-kDa protein was also present in frozen brain tumor samples, and the level of expression appeared to correlate with their corresponding primary cultures. Thus, our findings suggest that this 220-kDa protein may play an important role in determining $VD_3$ sensitivity in malignant glioma. BIOCHEM PHARMACOL **60**;9:1361–1365, 2000. © 2000 Elsevier Science Inc.

**KEY WORDS.** vitamin D; brain tumor; malignant glioma; vitamin D receptor; primary culture; apoptosis

Despite multimodality treatment for patients with primary malignant glioma, including new techniques for local radiation therapy [1], regional chemotherapy [2], immunotherapy [3], and recently gene therapy [3], survival remains poor. Since primary malignant glioma contains several types of receptors, such as epidermal growth factor receptor [4] and the VDR¶ [5], targeting these receptors may be another alternative strategy for the treatment of brain tumors. Using this approach, investigators have shown that $VD_3$, the vitamin D metabolite responsible for biological activity, can induce cell death in human malignant glioma cells [6, 7]. Furthermore, $VD_3$ has been demonstrated to have an antiproliferative effect in other types of tumor cells [8, 9]. The mechanism(s) of antitumor activity of $VD_3$ is not well understood. It has been postulated that $VD_3$ acts by binding to the VDR. The receptor–$VD_3$ complex then can modulate the transcription of a wide variety of genes that control cellular proliferation and differentiation. On the other hand, Magrassi *et al.* [7] have shown that 25-hydroxyvitamin $D_3$, which has low affinity for the VDR, has similar effects on glioma cells. This observation suggests that some important biological effects of vitamin D are not mediated through the classical receptor pathway. However, regardless of which mechanisms are involved, not all tumor cells are sensitive to $VD_3$. In this study, we investigated certain biochemical features that make these glioma cells sensitive to $VD_3$ and report our findings herein.

## MATERIALS AND METHODS

### *Materials*

The glioma cell lines U-373, U-118, T98G, and SW-1783 were obtained from the ATCC. Glioma 1 was established in our laboratory from a patient with grade III astrocytoma. All cell lines were maintained with Eagle's Minimum Essential Medium supplemented with 10% fetal bovine serum. T47D, a breast cancer cell line known to possess VDR, also was purchased from the ATCC.

### *Primary Tumor Cell Culture*

Fresh brain tumor samples were obtained and placed immediately in DMEM. The tumor samples were cut into small pieces, ground with a small pestle and passed through a 0.45-μm tissue sieve, washed with DMEM, and centrifuged. The pellet was divided into two portions. One portion was plated on a 25-$cm^3$ flask with DMEM supplemented with 10% fetal bovine serum, and the other was placed in DMEM supplemented with N2 (nerve growth factor obtained from GIBCO-BRL) and selenite. We used two types of medium since we found that for certain tumors containing an abundance of fibroblasts, it was best to seed the cells in serum-free medium. Cells usually started to

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¶*Abbreviations*: VDR, vitamin D receptor; $VD_3$, 1,25-dihydroxyvitamin $D_3$; and DMEM, Dulbecco's Modified Eagle's Medium.

Received 30 December 1999; accepted 14 April 2000.

**TABLE 1. Cytotoxicity of $VD_3$ in glioma cell lines and primary cultures**

| Sample | Cell lines | Tumor histology | $IC_{50}$ (nM) |
|---|---|---|---|
| 1 | U-118 | Cell line from a glioblastoma patient, ATCC | 60 |
| 2 | SW-1783 | Cell line derived from a grade III astrocytoma, ATCC | 37 |
| 3 | U-373 | Cell line from glioblastoma, ATCC | > 100 |
| 4 | T98G | Cell line from a glioblastoma patient, ATCC | > 100 |
| 5 | Glioma 1 | Cell line derived from a grade III astrocytoma | 23.5 |
| 6 | Primary culture | Grade III + oligodendroglioma | 20.6 |
| 7 | Primary culture | Grade III + oligodendroglioma | 35 |
| 8 | Primary culture | Grade IV or glioblastoma | 65 |
| 9 | Primary culture | Grade IV or glioblastoma | 67 |
| 10 | Primary culture | Grade IV or glioblastoma | 69.7 |
| 11 | Primary culture | Grade IV or glioblastoma | > 100 |
| 12 | Primary culture | Normal astrocyte | > 100 |

The $IC_{50}$ values represent an average of three experiments in established cell lines and two for primary cultures.

attach at 48–96 hr. Media were changed every 3–4 days. After 6–8 weeks, fibroblasts usually began to senesce, and after 10–12 weeks, when cultures were composed mostly of tumor cells, they were used to study the biological effect of $VD_3$. Glial fibrillary protein immunofluorescence staining was carried out to ensure that these cells were glial cells.

### *Growth Inhibition Assay*

Cells ($1 \times 10^4$) were seeded onto 6-well plates and allowed 8 hr for attachment. Various concentrations of $VD_3$ (purchased from Abbott) were added to each well. Media were changed every 2 days, and fresh $VD_3$ was added. After 6 days of exposure, viable cells were counted in the presence of 0.2% trypan blue. The $IC_{50}$ was determined by plotting the number of viable cells as a percentage of control against drug concentration.

### *Apoptosis Assay*

Cells ($1 \times 10^4$) were seeded onto Lab-Tek chamber slides (NALGE NUNC International) and then exposed to $VD_3$ for 72 hr. The apoptotic cells were detected by an *in situ* end-labeling assay using a kit from ONCOR. Briefly, cells were fixed in 4% neutral buffered formalin, treated with 2% hydrogen peroxide, and incubated with terminal deoxynucleotide transferase enzyme and digoxigenin-11-dUTP under a plastic coverslip for 1 hr. Anti-digoxigenin peroxidase was applied to the slide, followed by the chromogenic substrate diaminobenzidine and counterstained with hematoxylin. Cells that underwent apoptosis showed dark brown staining in the nuclei.

### *DNA Fragmentation Assay*

Cells ($5 \times 10^5$) exposed to $VD_3$ for 72 hr were harvested and centrifuged. DNA was extracted and electrophoresed on 1% agarose gels in TAE buffer (40 mM Tris acetate, pH 8.0, and 2 mM EDTA). The DNA fragmentation was visualized by staining with ethidium bromide (for detailed procedures, see Ref. 10).

### *Preparation of Subcellular Fractions*

Cells were resuspended in Tris low salt buffer containing 0.1% NP-40, 50 mM Tris–HCl, pH 8.0, 50 mM NaCl, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/mL of leupeptin and pepstatin, and 0.01 U of aprotinin. After brief agitation, the lysed cells were centrifuged at 4° for 1 min at 10,000 *g*, and the supernatant containing cytosolic and solubilized membrane proteins was removed and used as the cytosolic fraction. The pellet was resuspended in 150 μL of NP-40 high salt buffer (similar to the above buffer but with 450 mM NaCl), agitated briefly, incubated on ice for 10 min, and centrifuged. The supernatant was collected and is referred to as the nuclear fraction. Protein assay was done using a kit from Bio-Rad.

### *Western Blot Analysis*

Fifty micrograms of protein (either nuclear or cytosolic) was separated by SDS–PAGE and transferred onto nitrocellulose membrane, blocked with 5% non-fat dry milk for 15 mins, then incubated with VDR monoclonal antibody for 12 hr (one monoclonal antibody was purchased from Chemicon, and the other was secreted by a hybridoma cell line, XVIE6EG10, from the ATCC), washed, and incubated further with horseradish peroxidase-conjugated second antibodies. The immunoblot was detected by chemiluminescence, and the signal intensity was quantified by computerized image analysis as described in our previous publication [11].

## RESULTS

The growth inhibitory effects of $VD_3$ in cell lines and in primary brain tumor cell cultures are shown in Table 1. It is quite obvious that only certain glioma cells were sensitive to $VD_3$ ($IC_{50}$ = 20–40 nM), whereas other glioma cells were quite resistant ($IC_{50}$ > 100 nM) and some were intermediate ($IC_{50}$ = 41–70 nM) in sensitivity to $VD_3$. Since several investigators have demonstrated that $VD_3$ exerts its cytotoxic effect via induction of apoptosis, we

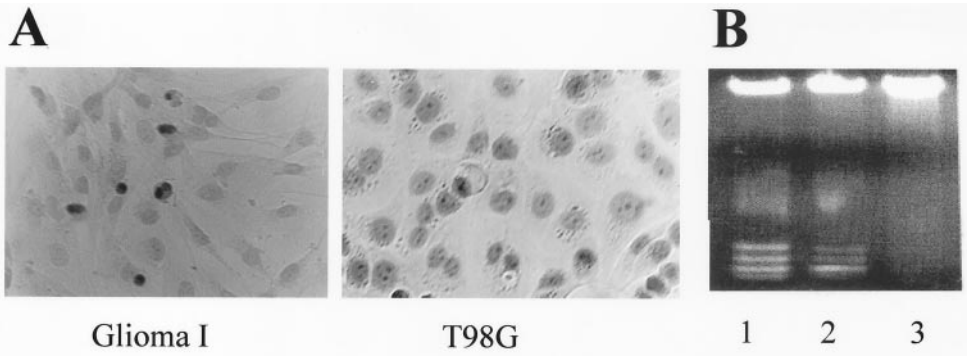


**FIG. 1. (A) *In situ* apoptosis detection in the Glioma 1 cell line and T98G after exposure to 45 nM (Glioma 1) or 100 nM (T98G) $VD_3$ for 24 hr. Glioma 1 cells that underwent apoptosis showed brown (dark) staining in the nuclei. T98G cells that are resistant to VD cells showed no dark staining. (B) DNA fragmentation study in three glioma cell lines after treatment with $VD_3$. Lane 1: Glioma 1; lane 2: U-118; and lane 3: U-373. DNA from untreated cells did not show fragmentation (data not illustrated).**

compared the apoptotic effect of $VD_3$ in four established cell lines (Glioma 1, U-118, U-373, and T98G) with their differential sensitivity to $VD_3$. DNA fragmentation and *in situ* labeling were used to study apoptosis, as described in Materials and Methods. Our data showed that $VD_3$ can induce apoptosis in cell lines that are sensitive to $VD_3$, i.e Glioma 1 and U-118 but not U-373 and T98G (the results of Glioma 1 and T98G are shown in Fig. 1A). Glioma 1, which is more sensitive than U-118, also showed a greater extent of DNA fragmentation (Fig. 1B). These findings suggest that the antitumor effect of $VD_3$ is due most likely to induction of apoptosis. Nevertheless, it remains unknown which factor(s) contributes to the apoptosis induced by $VD_3$.

To further clarify whether the apoptotic process is mediated by VDR, we assayed this receptor by western blot using two monoclonal antibodies specific for VDR, as described in Materials and Methods. T47D, a breast cancer cell line known to contain VDR, was used as a positive control. The results were as follows: When nuclear fractions were used to identify VDR, none of the glioma cell lines showed signal intensity at 54 kDa, which is the molecular mass of VDR, whereas T47D expressed an intense band (Fig. 2A). However, when cytosolic fractions were used, cell lines that were sensitive to $VD_3$ exhibited a band at 220 kDa, which was recognized by both monoclonal antibodies to VDR (Fig. 2B). It should be noted here that none of the cell lines expressed a 54-kDa band. Interestingly, normal astrocytes derived from fetal brain (provided by Dr. M. McCarty) did not show this 220-kDa protein. These cells were also resistant to $VD_3$ ($IC_{50} > 100$ nM). Furthermore, the amounts of this protein as estimated by a densitometer were correlated inversely with the $IC_{50}$ (Fig. 2C). Thus, it is possible that the 220-kDa protein may play a role in determining $VD_3$ sensitivity.

To investigate whether this protein is intrinsically present in brain tumors and is not altered during the establishment of cell lines, we assayed seven frozen tumor samples for their 220-kDa protein expression (Fig. 2D). Tumor samples also showed variable amounts of this 220-kDa protein, from strongly expressed (lane 3) to no expression (lanes 2, 4, and 6). It is noteworthy that the levels of expression of this 220-kDa protein in tumor samples (lanes 1, 2, and 3, panel D) appeared to correlate with those found in their corresponding primary cultures (lanes 12, 11, and 10, panel B). However, the signal intensities in tumor samples were less (lanes 1 and 3, panel D) than those found in their corresponding primary cell cultures (lanes 12 and 9, panel B). This most likely is due to multiple cell types found in tumor tissue (e.g. endothelial cells, fibroblasts, and macrophages) that do not possess the 220-kDa protein. Thus, our data indicate that this 220-kDa protein is intrinsically expressed in tumors and is not induced exogenously by factors present in the cell culture medium.

To further determine the relationship of this unique 220-kDa protein and $VD_3$, we investigated whether exposure to $VD_3$ changes the expression of this protein. Figure 3 shows that exposure to $VD_3$ did not affect the expression of the 220-kDa protein in Glioma 1 cells. Nor did $VD_3$ induce the expression of this protein in T98G cells (data not shown).

## DISCUSSION

The active metabolite of vitamin D ($VD_3$) has been shown to possess important biological effects that are not mediated by the classical receptor pathway [12–14]. $VD_3$ can induce changes in membrane fluidity, turnover of phospholipids, changes in $Ca^{2+}$ flux, and activation of protein kinase C [15–17]. It has been assumed that these actions emanated from the initial internalization of $VD_3$ with membrane receptor or binding of protein(s) different from the classical nuclear receptor [18]. Indeed, the presence of a membrane protein that binds $VD_3$ has been reported [19].

In our study, we identified a 220-kDa protein that is present in variable amounts in brain tumor samples as well as their respective primary cultures and established cell lines from the ATCC. This 220-kDa protein was recognized by two different anti-VDR monoclonal antibodies. One is specific for the DNA binding domain, whereas the other is

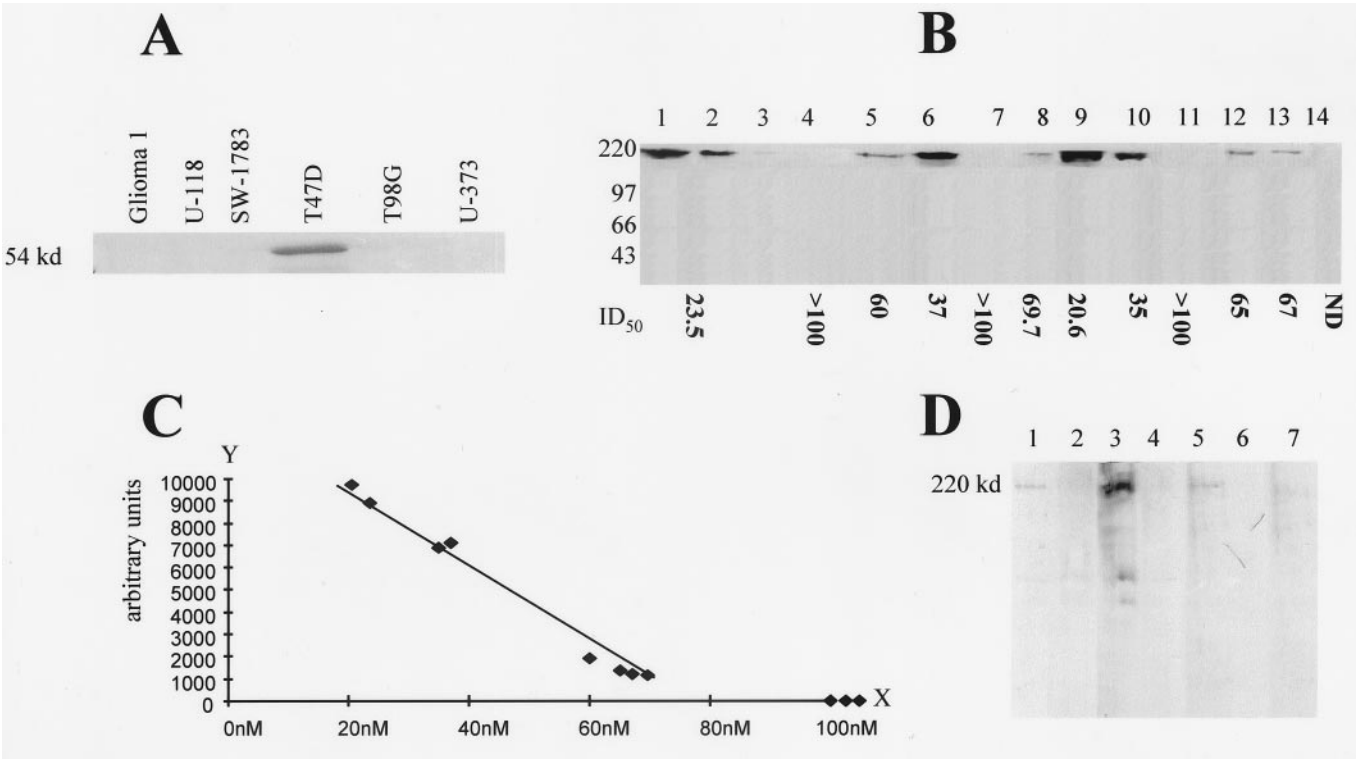


**FIG. 2. Western blot of glioma cell lines and primary cultures using anti-VDR antibodies. (A) No visible band for VDR appeared in the western blot analysis of nuclear protein from five glioma cell lines, whereas T47D cells, known to contain VDR, exhibited an intense band at 54 kDa. (B) Western blot analysis of glioma cell lines and primary cultures (see Materials and Methods and Results for details). The bottom label denotes the $IC_{50}$ values listed in Table 1. Lanes 1, 2, and 3: serial dilution of total protein of Glioma 1 cell lines. Lane 4: U-373; Lane 5: U-118; Lane 6: SW-1783; Lane 7: T98G; Lane 8: grade IV glioblastoma (GBM); Lane 9: grade III + oligodendroglioma; Lane 10: grade III + oligodendroglioma; Lane 11: Grade IV GBM; Lane 12: Grade IV GBM; Lane 13: Grade IV GBM; Lane 14: normal astrocytes. (C) The $IC_{50}$ (nM, X-axis) plotted against the level of the 220-kDa protein (arbitrary units, Y-axis). (D) Western blot analysis of brain tumor samples. Lanes 1, 2, and 3 are the tumor tissue samples from which the primary cultures in lanes 12, 11, and 10 of panel B were derived. Lanes 4–7 are samples of the frozen tumors from our tumor bank, and no primary cultures were derived.**

unknown [20]. However, this protein was not recognized by the anti-VDR polyclonal antibody against either the N- or C-terminal of VDR by immunohistochemical staining (data not shown). Thus, it is conceivable that this newly identified protein possesses a certain sequence homology to the DNA binding domain of VDR. It is also possible that this unique protein represents a post-translational modification of the VDR protein. This possibility appears unlikely, since this protein is much larger than VDR (220 vs 54 kDa). The other possibility is that this protein represents a chimera of two proteins in which one of the proteins contains a sequence similarity to the DNA binding domain of VDR. It should be noted that the DNA binding domain of VDR is a conserved region that is found in other members of the Steroid Thyroid Receptor Superfamily (STRS). While it is known that exposure to $VD_3$ can influence the expression of VDR, our study found no changes in the amounts of 220-kDa protein after exposure to $VD_3$. Nevertheless, our data strongly suggest that the presence of this protein plays an important role in determining the cellular sensitivity to $VD_3$.

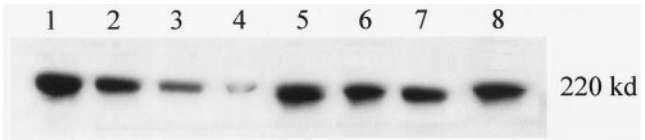


**FIG. 3. Western blot analysis of Glioma 1 cells after exposure to $VD_3$ at various times. Lanes 1–4, 10 μL (40 μg), 5 μL, 2 μL, and 1 μL of total protein extract of untreated Glioma 1 cells. Lanes 5–8, 10 μL of cell extracts from Glioma 1 cells treated with $VD_3$ for 1, 2, 3, and 4 days. Colloidal gold protein staining was performed to assure that similar amounts of protein were loaded on lanes 5–8 (data not shown).**

We also investigated whether this protein is found in tumors other than primary brain tumors. Our preliminary data showed that none of the 9 lung cancer cell lines (4 non-small cell lung cancers and 5 small cell lung cancers) possessed this 220-kDa protein. It is important to note that these cell lines were found not to be sensitive to $VD_3$ ($IC_{50}$ $> 100$ nM).

In this report, we also demonstrated that the antitumor effect of $VD_3$ is due most likely to induction of apoptosis, which is not mediated by VDR. This finding is in agreement with previous reports in the literature [6, 7, 21]. However, whether this effect is related to our newly

identified 220-kDa protein is not known and is currently being explored in our laboratory.

*This work is supported by P20CA60174 and the V.A. Research fund.*

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