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# Human Glioblastoma Cell Lines: Levels of Low-Density Lipoprotein Receptor and Low-Density Lipoprotein Receptor-related Protein[1]

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## ABSTRACT

**The status of the low-density lipoprotein (LDL) receptor and LDL receptor-related protein (LRP) in seven human glioma cell lines was evaluated to extend our knowledge of human glioblastoma multiforme tumor metabolism for future drug design. Cell lines SF-767, SF-763, A-172, U-87 MG, U-251 MG, U-343 MG, and SF-539 were used. Binding of $^{125}$I-labeled LDL to these cells at 4°C was carried out to determine the number of LDL receptors on cells and the affinity of LDL for these receptors. The content of LRP was measured by immunoblotting. The presence of specific saturable LDL receptors was proven in six of the cell lines investigated. SF-767 cells revealed high-affinity LDL binding (equilibrium dissociation constant, $K_d = 7$ nM) and maximum binding capacity approximating 300,000 receptors/cell. Most of the remaining cell lines had relatively lower affinity ($K_d = 38–62$ nM) but also had very high numbers of receptors (128,000–950,000/cell). All cell lines exhibited LRP, but the expression was variable. The cell lines SF-539, U-87 MG, and U-343 MG were particularly rich in this protein. The data suggest that glioblastoma cells have high numbers of LDL receptors; however, there is considerable variation in binding affinity. Overall, this finding suggests that LDL receptors on glioblastoma cells could potentially be useful for targeting antitumor agents. LRP, a multifunctional receptor expressed on glioblastoma cells, also has the possibility for serving as a therapeutic target.**

## INTRODUCTION

Malignant gliomas constitute 35–45% of primary brain tumors. Glioblastoma multiforme tumors are gliomas of highest malignancy (grade IV), characterized by uncontrolled, aggressive cell proliferation and infiltrative growth within the brain and general resistance to conventional treatment. Despite efforts to improve therapies or to develop new ones, the outcome of treatment for malignant gliomas is very modest (median survival after therapy is about 10 months; Ref. 1).

Increased cell proliferation and growth is associated with high turnover of cell cholesterol for membrane growth. Cells requiring cholesterol for membrane synthesis may take up plasma LDL[3], the main cholesterol carrier in blood, via receptor-mediated endocytosis (2), or they may initiate *de novo* synthesis of cholesterol. There is a body of evidence demonstrating that some types of cancer cells have high LDL requirements together with depletion of LDL in the blood of cancer patients. The latter is thought to be due to elevated LDL receptor levels on rapidly growing tumor cells (for review, see Ref. 3). Among types of tumors known to require high amounts of LDL are some types of leukemia (4, 5), cancers of gynecological origin (6), and lung tumor tissues (7, 8). It has been suggested that LDL may serve as a selective vehicle for delivery of therapeutic compounds into tumor cells.

There is a paucity of information on the presence and function of LDL receptors in brain cells, both normal and malignant (9–11). Using immunocytochemical techniques for LDL receptor localization in monkey and rat brain, Pitas *et al.* (11) reported relatively few LDL receptors in neurons and glial cells. Staining was most pronounced in astrocytes. Rudling *et al.* (9), using homogenates from human intracranial tumors and surrounding normal tissue, evaluated LDL receptor function by a $^{125}$I-labeled LDL binding assay. LDL receptor binding was highly variable between tumor tissue and normal brain tissue. These studies are potentially confounded by the possibility that the normal tissue surrounding the tumors may have undergone nonspecific changes due to edema or the release of cytokines.

We have previously reported (12) that boronated protoporphyrin that associates with LDL is endocytosed into the human glioblastoma cell line SF-767. Fluorescence microscopy demonstrated that the boronated protoporphyrin-LDL complexes were delivered to lysosomes, suggesting a LDL receptor mechanism. In the present study, we set out to demonstrate that uptake of LDL by SF-767 cells was by a high-affinity saturable LDL receptor mechanism and to estimate the number of LDL receptors on these cells. In the present study, we also determined whether the presence of high-affinity LDL receptors was a general feature of glioblastoma cells; therefore, six other cell lines were evaluated for their ability to bind LDL.

Another member of the LDL receptor family, LRP (13), is known to be involved in processes of cholesterol homeostasis in the central nervous system (for reviews, see Refs. 14 and 15). This multifunctional receptor endocytoses several structurally and functionally distinct ligands, including apo E, which can potentially target cholesterol to LRP-containing cells (16). In addition to determining the LDL receptor status of glioblastoma cells, we addressed the question whether these cells also possessed LRP; the latter was achieved by using immunoblotting techniques.

## MATERIALS AND METHODS

**Cell Culture.** Seven human glial tumor cell lines (SF-767, SF-763, A-172, U-87 MG, U-251 MG, U-343 MG, and SF-539) were obtained from the tissue bank of the Brain Tumor Research Center (University of California–San Francisco, San Francisco, CA) and have been described previously (17, 18). All of the lines originated from grade IV human glioblastoma biopsy specimens. The growth characteristics of the cell lines are shown in Table 1. The cells were grown in Eagle's MEM supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan, UT) and antibiotics [1% fungizone (Life Technologies, Inc., Gaithersburg, MD) and 50 mg/l gentamicin (Sigma Chemical Co., St. Louis, MO)]. Cultures were passaged twice a week to maintain the cells in exponential growth. Twenty-four h before carrying out LDL-binding studies, cells were switched to LPDS; under these conditions, doubling time was reduced 40–60%.

**Preparation and Radiolabeling of LDLs.** LDLs were isolated from plasma of healthy human subjects (obtained from the blood bank) by sequential ultracentrifugation (40,000 *rpm*, 4°C) using NaBr for density adjustment, as described (19). The LDL fraction (density, d 1.019–1.063 g/ml) was respun at 40,000 *rpm* to concentrate and further purify the LDL. LDL was dialyzed

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[3] The abbreviations used are: LDL, low-density lipoprotein; LRP, LDL receptor-related protein; LPDS, lipoprotein-deficient serum; apo, apolipoprotein.



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Table 1 *Human malignant glioma cell lines*

| Cell line | Diagnosis | Doubling time (days) | Passage no. |
|---|---|---|---|
| SF-767 | Recurrent glioblastoma multiforme | 1.2 | 134 |
| SF-763 | Recurrent glioblastoma multiforme | 1.1 | 202 |
| A-172 | Glioblastoma multiforme | 1.5 | 50 |
| U-87 MG | Glioblastoma multiforme | 1.3 | 144 |
| U-251 MG | Glioblastoma multiforme | 2.3 | 90 |
| U-343 MG | Glioblastoma multiforme | 1.5 | 10 |
| SF-539 | Gliosarcoma | 1.5 | 115 |

against PBS [10 mM phosphate buffer, 150 mM NaCl (pH 7.4), and PBS] at 4°C, and protein concentration was determined by the modified method of Lowry (20). The purity of isolated LDL was confirmed by SDS-PAGE (21).

LDLs were labeled with $^{125}$I (Amersham Pharmacia Biotech, Piscataway, NJ) using IODO-BEADS (Pierce Chemical Co., Rockford, IL) according to manufacturer's instructions, applying conditions according to Brown and Goldstein (22). Iodination was performed in 100 mM PBS (pH 6.5) for 20 min at room temperature. $^{125}$I-labeled LDL was then dialyzed in a Slide-A-Lyzer (Pierce Chemical Co.) against PBS (pH 7.4) and diluted with native LDL to give a final specific activity of about 500 cpm/ng. Both native and iodinated LDLs were kept refrigerated and used in binding experiments within 1 month.

**Preparation of LPDS.** After ultracentrifugation at d 1.215 g/ml, lipoproteins were removed by aspiration, and the remaining bottom fraction (*i.e.*, LPDS) was dialyzed against saline. After adjusting to initial volume, plasma was converted to serum by incubation for 24 h at 4°C with thrombin (final concentration 10 US units/ml). The resulting clot was removed by centrifugation. LPDS was sterile filtered and kept in aliquots at −20°C until used.

**$^{125}$I-labeled Binding to Glioblastoma Multiforme Cells.** Exponentially growing cells were seeded (day 1) at a concentration of approximately $1 \times 10^5$ cells/well into 6-well plates (35-mm wells; 2 ml of medium/well). On day 3, the cells were rinsed with PBS and then provided with fresh LPDS-medium [10% LPDS, 25 mM Hepes (pH 7.4), and 50 mg/l gentamicin in MEM]. Twenty-four h later (day 4), the binding experiments were carried out (the cells were 60–80% confluent).

The binding studies at 4°C were performed essentially as described by Innerarity *et al.* (23). Cells were precooled on ice for 15 min, and then the medium in each well was replaced with 1 ml of chilled (4°C) LPDS-medium containing varying concentrations of $^{125}$I-labeled LDL (in a range of 1–120 $\mu$g/ml) to measure total binding (in duplicate), or $^{125}$I-labeled LDL and 50-fold excess of native LDL (single well/concentration) to obtain information on nonspecific binding. The cells were incubated at 4°C for 4 h, after which the medium was removed (free $^{125}$I-labeled LDL) and cell monolayers were solublized after careful rinsing.

Cell protein was measured (20), and $^{125}$I-labeled LDL binding was determined by $\gamma$-counting (bound $^{125}$I-labeled LDL values). The difference between total binding and nonspecific binding provides information on the saturable, specific binding of LDL to cells.

The binding curves for SF-767 cells were linearized by Scatchard analysis (24), from which both $K_d$ (equilibrium dissociation constant) and $B_{max}$ (maximum binding capacity in ng/mg cell protein) were obtained. The number of receptors/cell was then calculated using molecular weight of apo B ~500 kDa and number of cells/well after binding. The data were expressed as means ± SE. Cell lines were first assayed for LDL binding in the range of 1–40 $\mu$g/ml $^{125}$I-labeled LDL on at least two separate occasions; cell lines not demonstrating saturation at 40 $\mu$g/ml were then assayed in the range of 2–120 $\mu$g/ml $^{125}$I-labeled LDL. The data reported here are those obtained with cells incubated with 2–120 $\mu$g/ml $^{125}$I-labeled LDL.

**Western Blot for LRP.** Glioma cells grown in LPDS medium were washed three times with PBS, harvested from flasks by scraping, and pelleted (yield about $5 \times 10^6$ cells/cell line). HepG2 cells (used as a LRP reference), grown as described previously (25), were also harvested. Cell pellets were frozen (−80°C) for later use.

Cells were lysed in 100 mM Tris-HCl buffer (pH 6.8) containing 2% SDS, 10% glycerol, and a mixture of protease inhibitors [5 mM phenyl-methanesulfonyl fluoride, 7 $\mu$g/ml aprotinin, and complete protease inhibitor mixture (1 tablet/10 ml; Boehringer Mannheim, Indianapolis, IN)]. Cell pellets were mixed vigorously for 10 min in lysis buffer, heated at 100°C for 5 min, and then centrifuged to obtain clear lysate. After protein determination (20), lysates were aliquoted and stored at −20°C.

Equivalent amounts of protein (20 $\mu$g/lane) were subjected to SDS-PAGE (21) under nonreducing conditions, using 4–20% gradient polyacrylamide gels (Novex, San Diego, CA). Proteins were transblotted to nitrocellulose and probed with anti-LRP antibody, which recognizes the 500-kDa extracellular portion of LRP (8G1), kindly provided by Dr. Dudley K. Strickland (American Red Cross, Rockville, MD). Rabbit liver membranes (26) were used as a standard to confirm the position of LRP. Antibody binding was detected by chemifluorescence (ECL Plus; Amersham Pharmacia Biotech), and relative intensity of bands was quantified using the Phosphor/fluor Imager (Bio-Rad Laboratories).

## RESULTS

The specific binding of LDL to SF-767 cells at 4°C is shown in Fig. 1*A*. The data indicate that saturation of LDL receptors is achieved at 20 $\mu$g/ml. $^{125}$I-labeled LDL binds to these cells with high affinity (Fig. 1*B*) with a calculated $K_d$ of 7 nM. From the Scatchard plot analysis we calculate that the maximum binding capacity is ~300,000 receptors/cell (Table 2). To determine whether other glioma cell lines

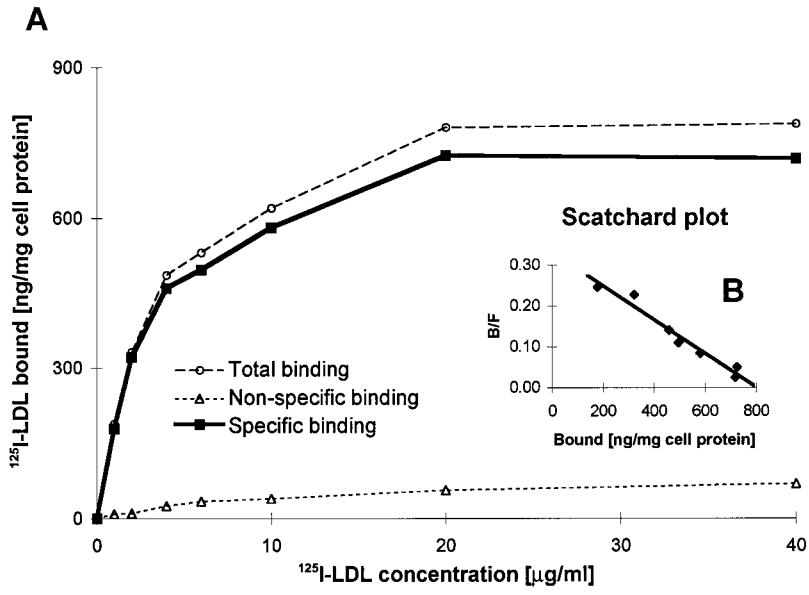


Fig. 1. $^{125}$I-labeled LDL binding to SF-767 cells. *A*, the cells were incubated at 4°C for 4 h with increasing amounts of $^{125}$I-labeled LDL in the absence (total binding) or presence (nonspecific binding) of 50-fold excess of unlabeled LDL. Specific binding was calculated by subtracting nonspecific from total binding. The figure is a representative example of four experiments. *B*, Scatchard plot of the specific binding. Bound/free represents $^{125}$I-labeled LDL bound (ng/mg of cell protein) divided by the amount of $^{125}$I-labeled LDL present in medium during binding (ng/ml). The plot indicates high-affinity binding ($K_d$ = 6.98 nM) of LDL to SF-767 cells.



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Table 2 *$^{125}$I-labeled LDL binding to glioblastoma multiforme cells*

| Cell line | No. experiments | $K_d$[a] ($\mu$g/ml) | $K_d$ (nM) | $B_{max}$ (ng/mg cell protein) | Number of LDL receptors/cell |
|---|---|---|---|---|---|
| SF-767 | 4 | 3.50 ± 1.05 | 6.98 ± 2.12 | 793 ± 105 | 288,000 ± 41,900 |
| SF-763 | 1 | 60.0 | 120 | 900 | 950,000 |
| A-172 | 1 | 62.0 | 124 | 1400 | 923,000 |
| U-87 MG[b] | 2 | — | — | — | — |
| U-251 MG | 2 | 38.0[c] | 76[c] | 225[c] | 128,000[c] |
| U-343 MG | 1 | 40.0 | 80 | 510 | 311,000 |
| SF-539 | 1 | 50.0 | 100 | 310 | 252,000 |

[a] $K_d$, a dissociation constant equal to concentration of $^{125}$I-LDL corresponding to half of maximal binding $B_{max}$ value; $B_{max}$, related to maximum binding capacity (for calculation, see "Materials and Methods").
[b] There was no evidence of binding in the range of 0–120 $\mu$g $^{125}$I-LDL/ml.
[c] Average value from two experiments.

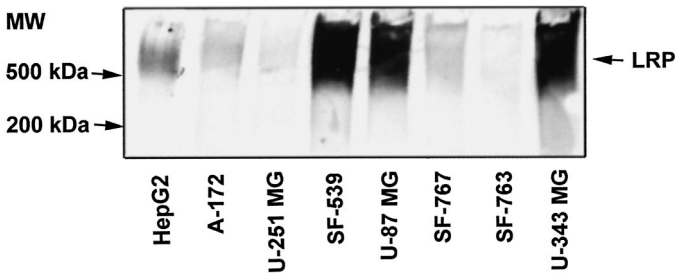


Fig. 2. Western blot analysis for LRP on HepG2 (used as a LRP-containing standard) and seven glioblastoma cell lines. Equivalent amount of cell lysates (20 $\mu$g protein/lane) were subjected to 4–20% SDS-PAGE under nonreducing conditions and transferred to nitrocellulose membrane. LRP was detected by an antibody directed against the external subunit of the receptor [1:1000 dilution in 10 mM Tris-HCl and 150 mM NaCl (pH 7.4), with 0.05% Tween 20] and revealed by immunofluorescence.

had similar LDL binding capacity as SF 767 cells, 4°C binding studies were carried out on the cells shown in Table 2. Compared with SF-767 cells, all other glioma cells had at least an order of magnitude lower binding affinity; however, two cell lines, SF-763 and A-172, had extremely high numbers of receptors (923,000–950,000 receptors/cell), suggesting these cells possessed large numbers of low-affinity receptors. Cell lines U-251 MG, U-343 MG, and SF-539 have receptor numbers more similar to that of SF-767 but have lower binding affinity than SF-767. One cell line, U-87 MG, had no detectable high-affinity receptors, and binding was solely nonspecific. To test whether U-87 MG cells express LDL receptor protein, cells were solublized and protein was electrophoresed on SDS-PAGE under reducing conditions and probed with anti-LDL receptor antibody (kindly provided by Dr. Janet Boyles). The U-87 MG cells were compared with human HepG2 cells known to have LDL receptor activity. Based on this analysis, U-87 MG cells had ~2-fold greater LDL receptor content than HepG2 cells (data not shown), indicating that, although specific binding was not evident, LDL receptor protein was present. During LDL-binding incubations, U-87 MG cells become rounded, suggesting that the lack of receptor activity may reflect a change in LDL receptor conformation in the morphologically altered cells.

It is well known that LRP functionally plays an important role in binding and uptake of apo E-containing lipoproteins (14). This receptor is present in large quantities in liver tissue; however, unlike the LDL receptor, it is not regulated by cholesterol in the medium. We asked the question whether LRP is present in glioblastoma cells and whether, like LDL receptors, there were major differences in the amount of receptor protein. The latter was evaluated by immunoblotting; a representative immunoblot ($n = 5$) is shown in Fig. 2. It is very clear that there are major differences between cell lines and that SF-539, U-87 MG, and U-343 MG are particularly rich in this protein. Table 3 summarizes the relative intensity of the LRP bands compared to the reference cell line, HepG2. The relative amount of LRP in SF 767 cells is similar (87%) to that of HepG2 cells, whereas U-87 MG, U-343 MG, and SF-539 have 4–6-fold higher relative mass. The remaining cell lines (A-172, U-251 MG, and SF-763) have low levels of LRP. These data suggest considerable variability in LRP expression in glioma cell lines.

## DISCUSSION

Although cerebrospinal fluid does not contain significant amounts of LDL (27), there is evidence that LDL receptors are present in monkey and rat brain but localized primarily to astrocyte-rich regions (11). The expression of LDL receptors by brain cells and the presence of apo E- and apo A-I-containing lipoproteins in cerebrospinal fluid suggest that the central nervous system has a mechanism for lipid transport and cholesterol homeostasis similar to that of other tissues (28).

Large numbers of high-affinity LDL receptors have been described in primary culture of rat glial cells obtained from brains of rat pups, 1–2 days of age (28), suggesting that, in rapidly growing rat brains, cholesterol requirement is likely to be high as in the case of many tumor cells. Although there is a paucity of information on LDL receptor affinity and number on glioblastoma multiforme cells, earlier studies have demonstrated binding (29) and internalization (30) of LDL to U-251 MG cells. The present study unequivocally demonstrates that SF-767 cells possess large numbers of high-affinity LDL binding sites, thus explaining our previous observation of time- and concentration-dependent uptake of boronated protoporphyrin into lysosomes (12). This compound is associated with LDL and is, thus, transported into cells via the LDL receptor. Our study also shows that glioblastoma cell lines, in general, seem to possess large numbers of LDL receptors, albeit there is a wide spectrum in binding affinity. We cannot rule out that some of this variability may be attributable to cell passage number. Alternatively, differences in binding affinity may reflect changes in plasma membrane lipid composition, which could alter LDL receptor conformation and subsequent LDL binding. The SF-767 cell line revealed high binding affinity comparable with the binding affinity described by Brown and Goldstein (22) for human skin fibroblasts, $K_d$ = 7.0 nM for SF-767 cell line *versus* 4.5 nM for fibroblasts. In conjunction with their high binding affinity, the SF-767 cells also had large numbers of LDL receptors, ~300,000/cell. In contradistinction to the SF-767 cells, the majority of glioblastoma

Table 3 *Relative amount of LRP on glioblastoma multiforme cells*[a]

| Cell line | Relative intensity (% of HepG2) |
|---|---|
| HepG2 | 100 |
| SF-767 | 87 |
| SF-763 | 22 |
| A-172 | 66 |
| U-87 MG | 471 |
| U-251 MG | 27 |
| U-343 MG | 530 |
| SF-539 | 622 |

[a] Quantification from Western blots, 20 $\mu$g of protein/lane.



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cells in the current study had relatively lower binding affinity, $K_d$ = 38–62 nM, but high numbers of receptors (128,000–950,000/cell). These $K_d$ values are comparable with $K_d$ of HepG2 cells (30 nM) (25). Our data suggest that the LDL receptor is up-regulated in glioblastoma cells, which is consistent with the observation that during early development of the rat, when glial cells are rapidly growing, there is a substantial increase in LDL receptors compared with adult glial cells, which have little or no LDL receptors (11, 28). Because our study indicates that glioblastoma cell lines have large numbers of receptors, the LDL receptor could potentially be useful for targeting antitumor drugs to glioblastoma cells using LDL as the transport vehicle.

LRP is a multiligand receptor, which has the capacity to bind and internalize apo E-containing lipoproteins and, hence, is an alternative route for the uptake of cholesterol into cells. Unlike the LDL receptor, which is not readily detectable in neurons and glial cells in normal brain (11), LRP is found in high abundance in neurons but not in glial cells (31–34). Interestingly, neoplastic transformation of glial cells is associated with an increased expression of LRP in glial cells compared with normal tissue (31). Moreover, Yamamoto *et al.* (35), demonstrated in neoplastic glioblastoma cells compared with low-grade astrocytomas that LRP expression was intense in the former cells and almost undetectable in the latter. This suggests that LRP expression may be regulated by the growth rate of the cells. It was previously reported that LRP is abundant in the glioblastoma cell lines U-87 MG (36) and A-172 and U-251 MG (35). Our studies confirm these findings and extend the observations to four additional cell lines (SF-767, SF-539, SF-763, and U-343 MG). Our data, together with that of others, strongly suggest that up-regulation of LRP may be a significant feature of glioblastoma cells. The present study indicates, however, that there is no apparent association between LDL receptor number and relative LRP expression because the cells with the highest number of LDL receptors (SF-763 and A-172) had relatively low LRP expression.

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