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Expression of α_2 Macroglobulin Receptor/Low Density Lipoprotein Receptor-Related Protein Is Cell Culture Density-Dependent in Human Breast Cancer Cell Line BT-20

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α_2Macroglobulin receptor/low density lipoprotein receptor-related protein (α_2MR/LRP) is a multifunctional cell plasma membrane receptor that binds and facilitates the endocytosis of a number of ligands involved in protease regulation and lipoprotein metabolism. In the invasive breast cancer cell line BT-20 we show that the expression of α_2MR/LRP can be strongly affected by cell culture density. By comparing measurements of mRNA, total cellular α_2MR/LRP antigen, and cell surface α_2MR/LRP expression we have demonstrated a marked cell density-dependent regulation of this receptor expression. Increasing the cell density results in a 3.4-fold increase in cell surface α_2MR/LRP expression. This corresponds to a marked increase in α_2MR/LRP mRNA in Northern blots of total RNA from cells cultured at high density and to consistent increases in α_2MR/LRP heavy chain in Western blots of cell lysates from high density cultures. These studies together demonstrate the significant up-regulation of α_2MR/LRP expression in BT-20 by increased cell density.

The α_2Macroglobulin receptor/low density lipoprotein receptor-related protein (α_2MR/LRP) is the largest known endocytic receptor. The receptor's ~600 kDa precursor protein is cleaved into a M_r 515 kDa heavy chain and a M_r85 kDa light chain (1, 2). The 85 kDa chain constitutes a transmembrane domain (2) and the 515 kDa chain is bound to it by non-covalent interactions. The heavy chain has binding sites for a number of ligands of a variety of structural groups and diverse functions (3, 4). Apart from activated complexes of α_2Macroglobulin, many other ligands can be internalised upon binding to α_2MR/LRP including ApoE, lipoprotein lipase, urokinase-type and tissue-type plasminogen activators and complexes between these and plasminogen activator inhibitor, *Pseudomonas* exotoxin A, and receptor-related protein (RAP) (3, 4). The important physiological role of α_2MR/LRP is illustrated by the fact that mice deficient for this receptor die early during gestation (5, 6).

α_2MR/LRP has been demonstrated in many types of cells including hepatocytes (7, 8), fibroblasts (9, 10), monocytes/macrophages (10, 11, 12), smooth muscle cells (13), and trophoblasts (14). However, in human tissues overall, α_2MR/LRP is mainly expressed in the placenta, liver and nervous tissues (1, 15).

The level of α_2MR/LRP varies between human tumour cell lines, and it seems to be characteristic of each tumour cell type and stage (9, 16, 17). For example, it is reported that α_2MR/LRP is present in photodynamic therapy-sensitive tumour cells and not in therapy-resistant cells (18), and that invasive breast and prostate cancer cells have lower α_2MR/LRP expression than do non-invasive cells (19). In other human tumours, α_2MR/LRP is decreased or absent in hepatocellular carcinoma (20) and in late stages of cutaneous melanocytic tumour progression (21), though it is increased in glial brain tumours (22, 23) and prostate carcinoma (24). In addition, up-regulation of α_2MR/LRP has been demonstrated in a human hepatocellular carcinoma line by adding dexamethasone (25).

In an earlier report we described the expression of α_2MR/LRP in the invasive human breast cancer cell line BT-20 (16), and in the present study we investigate the effect of cell culture density as a factor regulating the expression of α_2MR/LRP in this cell line.

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MATERIALS AND METHODS

Cell culture. The human breast cancer cell line BT-20 was kindly supplied by Mr T. Hurst (Royal Brisbane Hospital, Brisbane, Australia). All media and supplements for cell culture were obtained from Sigma Chemical Co., except for foetal bovine serum (FBS), which was supplied by Trace Biosciences (Castle Hill, NSW, Australia). The BT-20 cells were maintained in RPMI-1640 supplemented with 10% heat-inactivated FBS, 6 mM L-glutamine and 20 mM HEPES with 100 u/ml penicillin G and 100μg/ml streptomycin sulphate at 37°C in a 5% CO_2 atmosphere. The cells were passaged routinely every third day when they reached 80-90% confluency with a brief treatment in 0.05% trypsin / 0.02% EDTA in calcium- and magnesium-free Hank's balanced salt solution. Initial and final cell culture densities were determined by counting with a haemocytometer.

Quantitation of cell surface α_2MR/LRP by flow cytometry. The mouse monoclonal IgG1 antibody to human α_2MR/LRP (clone 8G1) was kindly provided by Dr Dudley Strickland of the American Red Cross, Rockville, MD, USA. This antibody is specific for the 515 kDa chain of α_2MR/LRP and it has been well characterised for use in immunohistochemistry, Western blotting and flow cytometry (14, 16, 22, 26). Flow cytometric quantitation of cell surface α_2MR/LRP was performed as described previously (16). Briefly, each cell suspension (50μl) was incubated with a saturating concentration of anti-human α_2MR/LRP antibody 8G1 at 4°C for 45 min. Control samples were incubated with the same amount of an unrelated monoclonal IgG1 isotype preparation (Becton Dickinson, San Jose, CA, USA). The cells were then washed and incubated in the dark at 4°C for 45 min with a saturating concentration of a secondary antibody, goat anti-mouse immunoglobulin, labelled with FITC (Becton Dickinson, San Jose, CA, USA). After washing, the cells were analysed by flow cytometry on a Becton Dickinson FACScan with a 488 nm Argon laser using predetermined instrument settings. Median fluorescence values were determined from the FL1 histogram for unstained (isotype control) and for stained cells, and the quantity of bound antibody was determined from the standardised Quantum Simply Cellular bead calibration plot (Flow Cytometry Standards Corporation, San Juan, Puerto Rico). The bead standards consist of four populations of microbeads coated with goat anti-mouse antibody, each population containing beads which bind a different number of mouse IgG molecules (3475, 11821, 44920 and 183072 molecules), as well as a blank population lacking antibody. The QSC beads were stained in exactly the same way as the tumour cells. A histogram of green fluorescence (FL1) was produced for the gated data and each peak of fluorescence corresponding to a bead type was identified by a specific histogram marker. The peak fluorescence channel number was taken, and a regression curve of channel number against binding capacity was constructed. A linear regression equation for the line was calculated using quickcal, the calibration software provided with the beads, and the number of antibodies bound to the tumour cells was determined by interpolation in this equation.

Western blot analysis. Cells were lysed in 125 mM NaCl, 10 mM Tris-HCl pH 7.4 containing 1 mM EDTA, 1 mM phenylmethylsulphonyl fluoride, 5μg/ml leupeptin, 5μg/ml aprotinin, 1% Triton X-100, and non-solubilized material was removed by centrifugation for 10 min at 6000g. Equal quantities of protein (60μg) were subjected to 7% SDS-polyacrylamide gel electrophoresis under non-reducing conditions. The proteins were then electrotransferred to a 0.45μm pore size nitrocellulose membrane (Bio-Rad, CA94547, USA) for 5 h at 100V in 20 mM Tris-HCl pH 7.2 containing 192 mM glycine and 20% methanol. The membranes were blocked with 5% nonfat milk for 1 hour, washed and incubated with 8G1 for 1 hour at 37°C. Antibody binding was detected with goat anti-mouse IgG (H+L) conjugated to horseradish peroxidase (Bio-Rad) using the ECL system (Amersham Life Science, England).

Northern blot analysis. Total RNA was isolated from BT-20 cells using the RNeasy Total RNA kit as described by the manufacturer (Qiagen, Germany). Equal amounts of total RNA (8μg) from each culture were denatured in formaldehyde, electrophoresed in 0.8% agarose gels, and blotted onto nylon membranes. The RNA was cross-linked on the membranes by ultraviolet irradiation and then pre-hybridised in 0.25M $NaPO_4$ buffer pH 6.5 containing 7% SDS, 50% formamide and 1mM EDTA at 48°C for 2 hours. The blots were probed with a 6.0 kb *Eco*RI/*Xho*I fragment of the human α_2MR/LRP cDNA, and with a 1.5 kb *Eco*RI/*Xho*I fragment of the mouse actin cDNA. (Clones of both these probes were obtained from the ATCC, Rockville, USA). The probes were labelled with [α^{32}P] dATP using a random oligonucleotide-primed synthesis kit (Promega, USA) and hybridised to membranes in prehybridisation solution for 24 hours at 48°C. After hybridisation the membranes were rinsed with 2 × SSC, 0.1% SDS, and washed twice for half an hour in the same solution at 48°C, and finally for half an hour in 0.2 × SSC, 0.1% SDS at 48°C. The hybridisation was quantitated by autoradiography using a Molecular Dynamics PhosphorImager with ImageQuant image processing software.

RESULTS

Cell surface expression of α_2MR/LRP. The effect of cell culture density on the cell surface expression of α_2MR/LRP was determined by flow cytometry. Culture vessels were seeded with BT-20 cells at a range of densities from 0.5×10^4 to 8×10^4 cells / cm^2. After culture for 4 days the final cell densities were counted and the cell surface α_2MR/LRP expression determined. Indirect fluorescent labelling of α_2MR/LRP-specific antibodies showed marked increases in cell surface fluorescence intensity by flow cytometry in high density cell cultures (see Figure 1). Quantitative determination of α_2MR/LRP surface expression by flow cytometry demonstrated up to 5-fold increases in expression with greater cell growth density (see Figure 2).

Western blot analysis of α_2MR/LRP. The total cellular α_2MR/LRP was estimated in Western blots of whole cell lysates using the α_2MR/LRP-specific monoclonal antibody 8G1. In each lysate a single band of the size expected for the 515 kDa heavy chain was produced. Significant increases in the intensity of the 515 kDa band were seen with increases in the cell culture density (see Figure 3).

Northern blot analysis of α_2MR/LRP mRNA. To see if the marked increases in cell surface and in total cellular α_2MR/LRP expression seen with greater cell culture densities coincide with higher α_2MR/LRP mRNA level, Northern blot analysis of extracts of total mRNA was undertaken. The cDNA probe hybridised to a single 15 kb RNA on Northern blots. Figure 4 shows the band intensities resulting from extracts from cells grown in high and low cell culture densities. From the Northern blots recorded on the PhosphorImager, the 15 kb band was analysed in the ImageQuant programme, and an average 4.85-fold increase in α_2MR/LRP mRNA was determined (see Table 1).

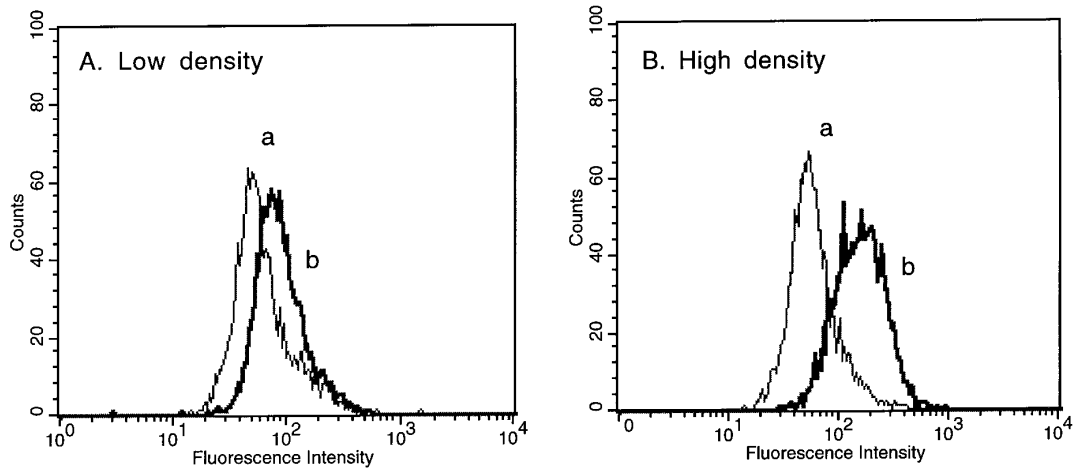

FIG. 1. Flow cytometric analysis showing cell culture density-dependent regulation of the cell surface expression of α2MR/LRP on BT-20 cells. Tumour cells were initially plated at low density (0.5×10^4 cells/cm^2) and at high density (8.0×10^4 cells/cm^2) and cultured for 4 days, changing the medium every 2 days. Cells were then harvested and the cell surface expression of α2MR/LRP was measured, obtaining signals using isotype-matched antibody control IgG1 (a) and α2MR/LRP monoclonal antibody 8G1 (b).

DISCUSSION

The results presented here clearly demonstrate marked increases in both the α_2MR/LRP and its mRNA with increases in cell culture density. There are numerous reports of both up- and down-regulation of α_2MR/LRP expression level in the literature, in addition to changes in its cellular distribution. Up-regulation of α_2MR/LRP levels has been observed in macrophages treated with colony stimulating factor-1 (27), in adipocytes treated with insulin (28) and in hepatoma HepG2 cells by dexamethasone (25). On the other hand, down-regulation has been shown in trophoblast cells by cyclic AMP (29), and in hepatocytes by propylthiouracil and by 17α-ethyloestradiol (30). Recent reports have also shown that down-regulation of α_2MR/LRP by lipopolysaccharide and by interferon-γ occurs in macrophages

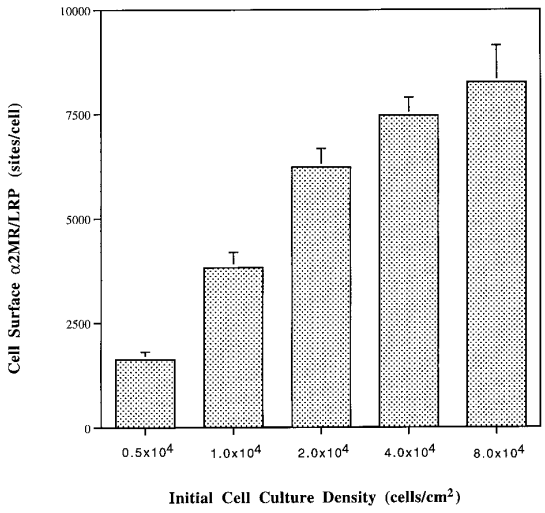

FIG. 2. The effect of cell culture density on BT-20 cell surface α2MR/LRP expression. Cells were plated at indicated initial culture densities and incubated for 4 days, changing the culture medium at 48 hour intervals. Cells were then harvested, the final cell culture densities were recorded, and cell surface α2MR/LRP expression was measured as described. The final cell culture densities were $5.24\pm0.88\times10^4$, $12.28\pm2.08\times10^4$, $21.08\pm3.96\times10^4$, $27.40\pm2.76\times10^4$, and $41.20\pm6.60\times10^4$ cells/cm^2, respectively. Data are means$\pm$SD of triplicate flasks.

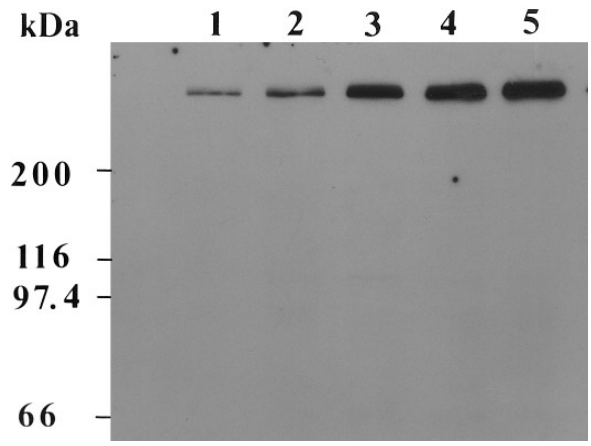

FIG. 3. Western blot analysis of α2MR/LRP. BT-20 cells were plated at different initial densities. Cells were incubated for 4 days, and α2MR/LRP 515 kDa in total cell lysates was detected by Western blots as described in Materials and Methods. Initial cell density in lane 1, 0.5×10^4 cells/cm^2; lane 2, 1.0×10^4 cells/cm^2; lane3, 2.0×10^4 cells/cm^2; lane 4, 4.0×10^4 cells/cm^2; lane5, 8.0×10^4 cells/cm^2. Molecular mass markers are indicated at the left. The data shown are from one of three independent experiments with similar results.

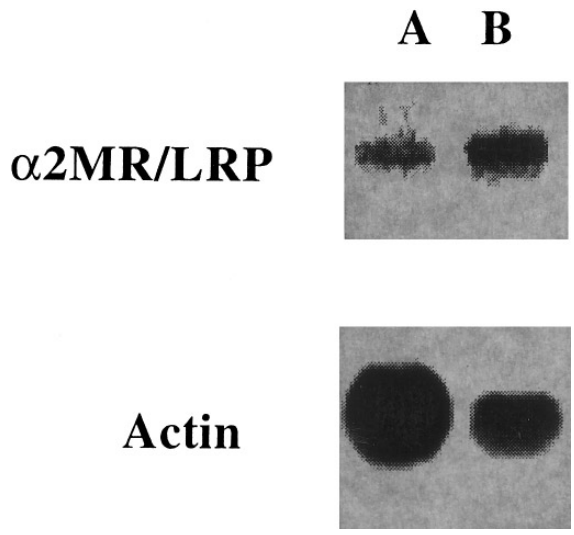

FIG. 4. Northern blot analysis of α2MR/LRP mRNA. BT-20 cells were plated at initial culture densities of 0.5×10^4 (A) and 8×10^4 cells/cm^2 (B). After 4 days incubation, total RNA was prepared from the harvested cells. α2MR/LRP and actin levels were estimated by Northern blotting as described in Materials and Methods.

and the RAW 264.7 macrophage-like cell line (31, 32). Epidermal growth factor and platelet-derived growth factor-BB have both been shown to induce a change in the subcellular distribution of α_2MR/LRP in vascular smooth muscle cells (33). These changes in expression level are all induced by soluble factors and pharmaceuticals. The present study demonstrates for the first time that α_2MR/LRP can also be up-regulated by cell culture density.

At the cellular level, several distinct mechanisms might be responsible for the observed regulation of α_2MR/LRP expression as a function of cell culture density. These may include the secretion of activators of α_2MR/LRP expression by BT-20 cells at high culture density, or perhaps the secretion of inhibitors of α_2MR/LRP expression at low cell culture density. It is also possible that α_2MR/LRP expression may be increased as BT-20 cells withdraw from the cell cycle at high culture density. The increase in cell-cell contact at high cell culture densities is also a likely mechanism for signaling the increase in α_2MR/LRP expression. The changes we have observed in α_2MR/LRP expression might be the result of some or all of these cellular mechanisms operating simultaneously.

The role of regulation by the cellular environment of the expression of the α_2MR/LRP gene has important implications for our understanding of the mechanisms of cancer progression. α_2Macroglobulin (α_2M), a ligand of α_2MR/LRP, is a large proteinase inhibitor of the blood plasma and extracellular tissue fluids (34). Upon reaction with a proteinase or reactive amine moiety, α_2M undergoes a conformational change which enables it to be recognised by and bound to the α_2MR/LRP, when it is referred to as 'activated α_2M' (α_2M*) (34, 35, 36). α_2MR/LRP has been identified as a receptor for cytokines bound to α_2M* (37), and these may be targeted to cells expressing α_2MR/LRP. It has been shown that this process results in the rapid clearance of complexes of α_2M* with transforming growth factor β1, with tumour necrosis factor α, and with platelet-derived growth factor-BB (38, 39, 40), and that inhibition of PDGF-BB-induced fibroblast proliferation by plasmin-activated α_2M* is also mediated through an α_2MR/LRP-dependent mechanism (41). All these studies suggest that the level of expression of α_2MR/LRP on tumour cells may be an important determinant of the cells' responsiveness to cytokines.

The complex process of tumour metastasis involves a number of proteases including urokinase-type plasminogen activator, cathepsin B, and various metalloproteases (42, 43). Extracellular proteinases are essential for tumour cells to penetrate the basement membrane, a process that typically distinguishes a carcinoma *in situ* from an invasive carcinoma. Proteolytic degradation of the extracellular matrix is also required when invasive tumour cells penetrate tissue, gain access to blood circulation (intravasation), and exit blood vessels (extravasation) to colonise distant metastatic sites. It has been found that these extracellular matrix-degrading proteinases are associated with the invading edge of tumours (44, 45, 46, 47). The ability of α_2Macroglobulin to contain and to clear these proteases and protease-inhibitor complexes may be central to the process of tumour cell invasion and metastasis. Consequently, recognition and endocytosis of proteinases, proteinase inhibitors and proteinase-inhibitor complexes may also be an important function of α_2MR/LRP on tumour cells. The multifunctional receptor α_2MR/

TABLE 1

The Effect of Cell Culture Density on α2MR/LRP mRNA Level

Expt. No.[a]	Initial low cell culture density (0.5×10^4 cells/cm^2)	Initial high cell density (8×10^4 cells/cm^2)	Factor of increase
1	0.031[b]	0.099	3.33
2	0.026	0.151	5.85
3	0.107	0.248	2.32
4	0.008	0.063	7.88
Mean ± SD			4.85 ± 2.51

Note. BT-20 cells were plated at the indicated initial densities and incubated for 4 days. Total RNA was prepared from the harvested cells. α2MR/LRP and actin levels were estimated from Northern blots as described in Materials and Methods.

[a] Data from independent experiments.

[b] The ratio of α2MR/LRP to actin was obtained after the quantitation of counts in the Northern blotting bands.

LRP, which can bind and endocytose α_2Macroglobulin-protease complexes (26, 48, 49), urokinase-type plasminogen activator (50) and its complexes (51) could therefore have a key role in mediating their local effects, and its level of expression may be an important determinant of invasiveness. Evidence produced here indicated that increasing cell culture density is associated with increased α_2MR/LRP expression. The extrapolation of these results to the *in vivo* tumour microenvironment would suggest that individual cells or small groups of invading tumour cells distant from the main tumour mass would express less α_2MR/LRP, a feature which may be pivotal in determining their ability to invade.

Recent Studies on the involvement of α_2MR/LRP in the progression of various cancer types have produced inconsistent results, which in some cases show that it is decreased or absent in malignant tumours (9, 17, 19, 20, 21), whilst in others there is overexpression of the receptor (22, 24). In the breast cancer lines which we have examined there appears to be a correlation between α_2MR/LRP expression level and invasiveness, and this is an aspect that we are currently investigating.

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