Article

A clathrin/dynamin- and mannose-6-phosphate receptor–independent pathway for granzyme B–induced cell death

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The 280-kD cation-independent mannose-6-phosphate receptor (MPR) has been shown to play a role in endocytic uptake of granzyme B, since target cells overexpressing MPR have an increased sensitivity to granzyme B–mediated apoptosis. On this basis, it has been proposed that cells lacking MPR are poor targets for cytotoxic lymphocytes that mediate allograft rejection or tumor immune surveillance. In the present study, we report that the uptake of granzyme B into target cells is independent of MPR. We used HeLa cells overexpressing a dominant-negative mutated (K44A) form of dynamin and mouse fibroblasts overexpressing or lacking MPR to show that the MPR/clathrin/dynamin pathway is not required for granzyme B uptake. Consistent with this observation, cells lacking the MPR/clathrin pathway remained sensitive to granzyme B. Exposure of K44A-dynamin–overexpressing and wild-type HeLa cells to granzyme B with sublytic perforin resulted in similar apoptosis in the two cell populations, both in short and long term assays. Granzyme B uptake into MPR-overexpressing L cells was more rapid than into MPR-null L cells, but the receptor-deficient cells took up granzyme B through fluid phase micropinocytosis and remained sensitive to it. Contrary to previous findings, we also demonstrated that mouse tumor allografts that lack MPR expression were rejected as rapidly as tumors that overexpress MPR. Entry of granzyme B into target cells and its intracellular trafficking to induce target cell death in the presence of perforin are therefore not critically dependent on MPR or clathrin/dynamin-dependent endocytosis.

Introduction

Cytotoxic T lymphocytes (CTLs)* and natural killer cells induce contact-dependent target cell death to protect higher organisms from overwhelming viral infection and cellular transformation. Extensive studies in gene-targeted mice indicate that these cytotoxic lymphocytes (CLs) rely principally on the exocytosis of toxins from specialized granules stored in their cytoplasm to kill unwanted or harmful cells (Barry and Bleackley, 2002). The two essential components of CL secretory granules are perforin, a membrane-disrupting protein, and a family of serine proteases called granzymes, which induce apoptosis in a perforin-dependent manner (Barry and Bleackley, 2002). Granzyme B is the most efficient proapoptotic granzyme (Shi et al., 1992) and the only mammalian serine protease that can mimic caspases by cleaving substrates at acidic residues, particularly aspartic acid (Odake et al., 1991). Congenital deficiency of granzyme B from CL results in delayed DNA fragmentation in target cells (Heusel et al., 1994) and marked susceptibility of the deficient mice to the viral pathogen ectromelia (Mullbacher et al., 1999). The precise mechanism of perforin/granzyme B collaboration is still not completely clear. Granzyme B is known to enter cells in the absence of perforin through receptor-mediated endocytosis into Rab 4/5-positive early endosomes (Shi et al., 1997; Pinkoski et al., 1998) but is innocuous to cells unless

perforin is also present. It is now becoming clear that perforin's role is to liberate granzyme B from the endosomal compartment into the cytosol (Browne et al., 1999) where it accesses and cleaves the key substrate Bid, a proapoptotic member of the Bcl-2 family, to initiate apoptosis through the mitochondrial pathway (Barry et al., 2000; Heibein et al., 2000; Sutton et al., 2000). Other pathways that operate independently of Bid also exist (Thomas et al., 2001), but they appear to be less efficient.

Although ^{125}I-labeled granzyme B's binding to the target cell surface is saturable and can be partly competed by unlabeled granzyme (Froelich et al., 1996), the mechanisms governing granzyme uptake have remained unclear. Recently, however, it was shown that granzyme B can enter cells after binding the 280-kD cation-independent mannose-6-phosphate (M6P) receptor (MPR) and that cells overexpressing MPR are more sensitive than parental cells to granzyme B–mediated death (Motyka et al., 2000). Granzymes have been known to bind to intracellular MPR for some time in the context of the trafficking of nascent granzyme polypeptide from the Golgi to secretory (lysosome-like) vesicles of CL (Griffiths and Isaaz, 1993). The MPR is also known to traffic to the plasma membrane in many (although not all) cells. MPR's demonstrated ability to internalize granzyme B is significant because in the absence of an alternative means of entering target cells MPR would become a legitimate target to block CL-mediated cell death by pharmacological means, for example, to block CTL–induced tissue damage in certain autoimmune diseases. It has also been proposed that cancer cells down-regulating MPR expression from their plasma membrane might escape immune surveillance by CL, a potentially novel tumor escape mechanism (Motyka et al., 2000). The same mechanism might also account for the observation that MPR can act as tumor suppressor in certain cancers, such as human hepatocellular (De Souza et al., 1995), breast (Chappell et al., 1997), and renal (Morita et al., 1991) carcinomas. A further important claim in respect to MPR was that its expression is required for rejection of an allogenic tumor graft across a complete H-2 haplotype mismatch (Motyka et al., 2000). Clearly, such a finding, if corroborated, would be profoundly important for clinical organ transplantation.

Although it is clear that MPR is one mechanism through which granzyme B can enter the target cell, no study has yet determined the susceptibility of cells to granzyme B/perforin-induced cell death in the absence of a functional MPR pathway. Given the importance of granzyme-mediated death pathways in infectious and neoplastic diseases (Trapani and Smyth, 2002), we set out to examine whether the MPR pathway is critical for granzyme B–mediated cell death. In the present study, we show that the absence of the clathrin-dependent MPR endocytic pathway reduces but does not abolish granzyme B uptake and, importantly, has little effect on cell death. Furthermore, we found that MPR expression was not required for rejection of an alloreactive tumor from the subcapsular space of the kidney in immunocompetent BALB/c and C57BL/6 mice. Therefore, other constitutive mechanisms exist in most cells for uptake of granzymes that are independent of MPR-dependent endocytosis.

Results

It has been demonstrated recently that overexpression of MPR in mouse L cells makes them more sensitive to granzyme B–induced apoptosis (Motyka et al., 2000). However, no study has examined whether the absence of MPR compromises apoptosis in response to granzyme B, either in vitro or in vivo. High concentrations of the competitor monosaccharide M6P in the previous study failed to completely abrogate cell death through granzyme B, leaving open the possibility that alternative pathways exist for granzyme B uptake into target cells (Motyka et al., 2000). Therefore, we wished to determine whether cells in which granzyme B cannot be taken up through the MPR remain susceptible to granzyme B–mediated cell death.

Granzyme B uptake is slowed but not abolished in K44A mutant–dynamin–expressing HeLa cells

To address the above issues, we performed studies in two sets of stably transfected cell lines, human HeLa cells and the same mouse L cell fibroblasts (C3H and H-2^k) used in the previous report (Motyka et al., 2000). Molecules that bind to cell surface MPR (and most other cell surface receptors including the transferrin receptor) are endocytosed via clathrin-coated pits and vesicles (Pearse and Robinson, 1990). In the first set of experiments addressing the question of granzyme B uptake, we used a previously well-characterized transfectant HeLa cell line expressing a mutated dynamin (K44A) that is a dominant-negative inhibitor of clathrin-dependent endocytosis (Damke et al., 1994). Dynamin's normal function is to permit the separation of clathrin-coated pits from the plasma membrane into the cytoplasm, thus enabling the trafficking of vesicles and their extracellular cargo to the late endosomal compartment, and ultimately to lysosomes (Damke et al., 1994). Expression of K44A-dynamin in the HeLa cell populations is regulated by a tetracycline (tet)-sensitive promoter so that growth in tet-deficient medium resulted in overexpression of K44A-dynamin. As a control, wild-type dynamin was overexpressed in HeLa cells using the same promoter.

Expression of K44A-dynamin has been shown to result in retention of some ligands, including transferrin at the plasma membrane, whereas other ligands fail to accumulate on the cell surface (Damke et al., 1994). To demonstrate the induction of K44A-dynamin expression, cells grown in the presence of tet or in the absence of tet for 48 h were incubated at 37°C with FITC-labeled transferrin (FITC-transferrin) and then viewed by confocal laser scanning microscopy. As expected, HeLa cells in which K44A-dynamin expression was repressed by tet demonstrated strong uptake of FITC-transferrin into cytoplasmic vesicles. In addition, most of the cells demonstrated punctate fluorescence at the plasma membrane, indicating multifocal binding to the transferrin receptor (Fig. 1a). In cells grown in tet-deficient medium, the fluorescence was largely restricted to the plasma membrane and few vesicles were internalized, consistent with defective clathrin-dependent uptake of FITC-transferrin. Fewer than 10% of cells demonstrated clear vesicular uptake under these conditions after 120 min, although some residual fluorescence was seen in the cyto-
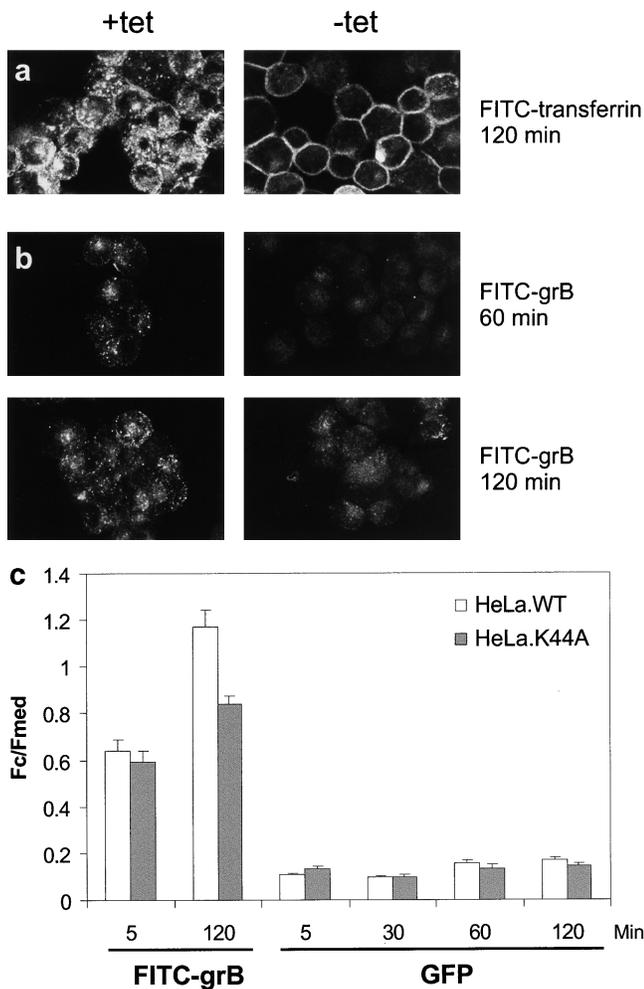
+tet   **-tet**

a   FITC-transferrin 120 min

b   FITC-grB 60 min

FITC-grB 120 min

Figure 1. **Residual uptake of granzyme B by K44A mutant dynamin–overexpressing HeLa cells.** (a) Confocal micrograph of HeLa cells overexpressing K44A mutant dynamin (−tet) or in which expression of mutant dynamin is suppressed (+tet) after exposure to FITC-transferrin for 120 min. (b) Confocal micrograph of K44A HeLa cells after exposure to FITC–granzyme B (50 nM) for 60 or 120 min. (c) Uptake of FITC–granzyme B (grB) or unglycosylated GFP by HeLa cells overexpressing wild-type (WT) or K44A-mutated dynamin for the times indicated. Uptake of either fluoresceinated molecule into the cell cytoplasm was quantitated by confocal laser scanning microscopy and image analysis as described (Jans, 1995; Jans et al., 1996). The results are expressed as a ratio between cytoplasmic fluorescence (Fc) and extracellular fluorescence (Fmed) ± standard error of the mean. Each data point was derived from measurements on at least 30 cells selected at random after substruction of autofluorescence (as described in Materials and methods).

plasm, consistent with constitutive uptake through fluid phase micropinocytosis, which is known to remain active in these cells (see below). Despite the demonstrated potent inhibition of receptor-dependent endocytosis in these cells (Damke et al., 1994), >90% of K44A-dynamin–expressing cells incubated with FITC–granzyme B showed cytoplasmic fluorescence, which increased with time (Fig. 1 b, −tet) and was far above levels of autofluorescence (see below). No cell surface binding of FITC–granzyme B was seen at either 4°C (unpublished data) or 37°C, and the cytoplasmic staining pattern was less obviously punctate than seen when only wild-type dynamin was expressed. These findings were again

consistent with fluid phase uptake of granzyme B. Control cells expressing only wild-type dynamin showed consistent cell surface and punctate vesicular staining with FITC–granzyme B similar to that seen with FITC-transferrin (Fig. 1 b, +tet) and reminiscent of granzyme B trafficking observed previously in Jurkat or FDC-P1 cells (Jans et al., 1996; Browne et al., 1999; Motyka et al., 2000). Similar HeLa cell transfectants that overexpressed wild-type dynamin upon withdrawal of tet showed normal vesicular uptake of both FITC–granzyme B and FITC-transferrin (Damke et al., 1994; data not shown).

The significant cytoplasmic fluorescence of K44A-dynamin–expressing cells exposed to granzyme B was confirmed and quantitated by laser scanning microscopy and image analysis (Fig. 1 c). As measured by the ratio of cytoplasmic to extracellular fluorescence (Fc/Fmed), cells overexpressing K44A-dynamin had taken up quantities of FITC–granzyme B equivalent to cells expressing only wild-type dynamin at the earliest time point that could practically be studied (t = 5 min). Over the next 2 h, the K44A-dynamin–overexpressing cells took up less granzyme B than control cells. A control unglycosylated fluorescent protein (GFP) that cannot bind to MPR was taken up equally well into K44A- or wild-type dynamin-expressing cells by fluid phase micropinocytosis. Overall, these results suggested rapid but low level constitutive fluid phase uptake of granzyme B (and GFP) together with more prolonged uptake of granzyme B through receptor-dependent endocytosis. Our results for granzyme B uptake were not explained by the uptake of free FITC or degraded FITC–granzyme B, since the cytoplasmic fluorescence was inhibited in a dose-dependent manner by coadding unlabeled granzyme B (see below). Using a polyclonal anti-MPR antiserum, we also showed that the HeLa cells expressed equivalent levels of MPR at the cell surface, irrespective of whether mutated or wild-type dynamin was expressed (unpublished data).

As described above, we postulated that the residual granzyme B uptake in K44A-dynamin–expressing cells was due to an alternative, clathrin/dynamin-independent uptake pathway. However, it was also plausible that the level of K44A-dynamin expression in the HeLa transfectants was insufficient to completely block granzyme B uptake through the clathrin-dependent MPR pathway. To distinguish between these possibilities, we incubated the K44A-dynamin–expressing cells with FITC–granzyme B in the presence of 5 mM mannose-6-phosphate, which represents a 100,000-fold molar excess of the monosaccharide over granzyme B (typically used at 25–75 nM). As a control, we added a similar concentration of glucose-6-phosphate (G6P), which is unable to bind to MPR (Motyka et al., 2000). As expected, uptake of FITC–granzyme B into HeLa cells expressing only wild-type dynamin was considerably reduced by M6P, whereas G6P had no effect (Fig. 2). By contrast, preincubation of cells with M6P did not diminish granzyme B uptake in K44A-dynamin–expressing HeLa cells, indicating that uptake of granzyme B through the MPR/clathrin pathway was already efficiently blocked in these cells. Overall, our results strongly suggested that granzyme B is able to enter the cell cytoplasm using a mechanism that is independent of the MPR/clathrin pathway.

Figure 2. **Granzyme B uptake into K44A mutant dynamin–overexpressing HeLa cells is not inhibited by a vast molar excess of M6P.** Cytofluorographic analysis of HeLa cells overexpressing K44A mutant dynamin (−tet, bottom) or expressing only wild-type dynamin (+tet, top) after incubation with FITC–granzyme B (50 nM) at 37°C for 20 min or in the absence of FITC–granzyme B (filled traces). Cells were preincubated either with 5 mM M6P (red) or G6P (blue) or without competing monosaccharide (green) for 15 min at 37°C.
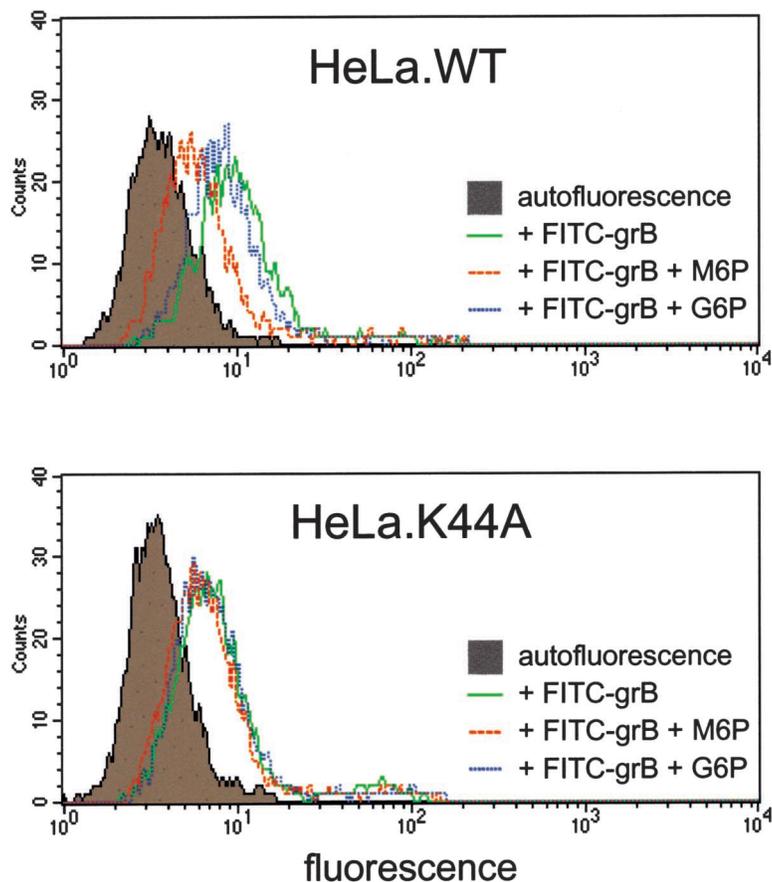
K44A-dynamin–overexpressing HeLa cells are not protected from granzyme B–induced cell death

We next determined whether K44A-overexpressing cells are protected from cell death mediated by granzyme B. We initially measured specific ^{51}Cr release in response to granzyme B delivered either by sublytic quantities of perforin or an alternative lytic agent, pneumococcal pneumolysin (PLO), which has been shown to closely mimic perforin's delivery of granzyme B (Browne et al., 1999). In this context, ^{51}Cr release is a measure of plasma membrane permeability during apoptosis and is a reliable short term measure of cell survival in response to granzyme B (Sutton et al., 1997; Trapani et al., 1998). Addition of granzyme B (50 nM) with either perforin or PLO induced equivalent ^{51}Cr release from K44A- and wild-type dynamin-overexpressing cells over 4 h (Fig. 3 a). This result is reminiscent of an experiment of Motyka et al. (2000) who showed that MPR-overexpressing and MPR-null L cells released equivalent ^{51}Cr when they were attacked by intact CTL. The authors attributed the surprisingly high ^{51}Cr release in cells lacking the MPR/clathrin pathway to perforin-mediated lysis and ruled out the effect as being caused by granzyme B. However, our experiment performed with the same cell lines and purified perforin and granzyme B clearly showed that, even in the absence of MPR, granzyme B is able to synergize with perforin or PLO to induce ^{51}Cr release. In this setting, the release of ^{51}Cr therefore reflects the loss of plasma membrane integrity as the result of granzyme B–induced apoptosis, not necrosis (lysis), in response to perforin. We also confirmed that K44A- and wild-type dynamin-

expressing cells were equally susceptible to granzyme B/perforin in assays of clonogenic survival. There was an equivalent dose-related reduction in colony numbers as the concentration of granzyme B increased, irrespective of whether the MPR/dynamin pathway was functional or not (Fig. 3 b).

Granzyme B uptake and cell death in MPR-null L cells

Our second series of experiments used mouse L cell fibroblasts either null for MPR expression or overexpressing human MPR as had been studied previously (Nolan et al., 1990; Motyka et al., 2000). The L cells overexpressing MPR (designated MS9-II) became strongly fluorescent after incubation for 10 min at 37°C with FITC–granzyme B and showed a similar pattern of fluorescence when viewed by confocal microscopy as described above for HeLa cells (Fig. 4 a). The staining pattern in most cells also clearly showed a predominant concentrated area of cytoplasmic fluorescence consistent with transport of ligand to the late endosomal compartment. The MPR-null MS cells (which had been transfected with vector DNA alone) were less strongly fluorescent at the same time point but still showed significant uptake of FITC–granzyme B compared with autofluorescence controls (Fig. 4 a). As was seen with the K44A-overexpressing HeLa cells, MS cells also showed minimal cell surface binding of FITC–granzyme B, whereas strong plasma membrane binding was seen in MS9-II cells. It was evident from kinetic studies in which FITC–granzyme B uptake was quantitated on a cytofluorograph that granzyme B uptake in MS cells could be partially compensated by longer incuba-
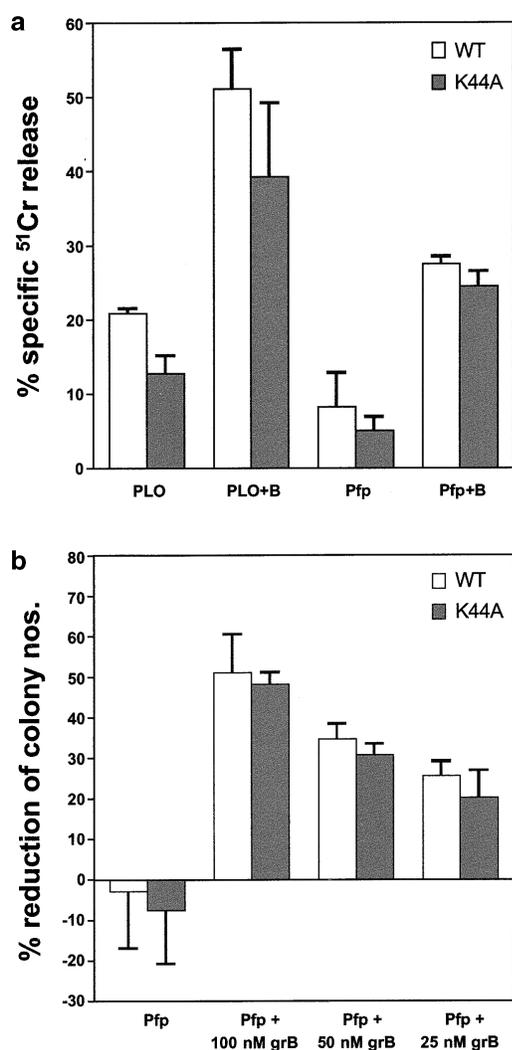
a

b

Figure 3. **K44A-dynamin–overexpressing HeLa cells are not protected from granzyme B–induced cell death.** (a) ^{51}Cr-labeled HeLa cells overexpressing either K44A- or wild-type (WT) dynamin were incubated with sublytic perforin (Pfp) or pneumolysin (PLO) either alone or together with granzyme B (50 nM) for 90 min at 37°C. Specific ^{51}Cr release is shown as the mean of triplicate data points ± standard error. The assay is representative of three similar experiments. (b) Clonogenic survival of K44A- or wild-type (WT) dynamin-overexpressing HeLa cells after incubation with sublytic perforin (Pfp) and various concentrations of granzyme B. Results are shown as the percentage inhibition of colony growth (mean of three cultures ± standard error at each granzyme concentration) compared with untreated cells. Typically, 75–150 HeLa colonies became evident when the cells were untreated. The experiment shown is representative of four similar experiments (as described in Materials and methods).

a

b

Figure 4. **Residual uptake of granzyme B by MPR-null L cell fibroblasts.** (a) Confocal micrograph of MS (MPR-null) and MS9-II (MPR-overexpressing) L cells after exposure to FITC–granzyme B (50 nM) for 60 min at 37°C or without addition of FITC–granzyme B (autofluorescence [AF], bottom). Association of granzyme B with the plasma membrane (m) and in the late endosomal compartment (l) are shown. (b) Cytofluorographic analysis of MS and MS9-II cells after incubation with FITC–granzyme B (50 nM) at 37°C for the times indicated or in the absence of FITC–granzyme B (filled traces).

tion. Whereas FITC–granzyme B uptake into MS9-II cells had reached maximal steady-state levels within 10 min, the fluorescence of MS cells had increased somewhat but still remained below that of MS9-II cells after 40 min (Fig. 4 b).

To characterize the residual uptake of granzyme B into MPR-null MS cells, we next performed a quantitative, kinetic analysis of granzyme B uptake using quantitative laser-scanning microscopy of several hundred cells. Cells were preincubated with FITC–granzyme B at 4°C to allow surface binding and then rapidly transferred to 37°C to per-
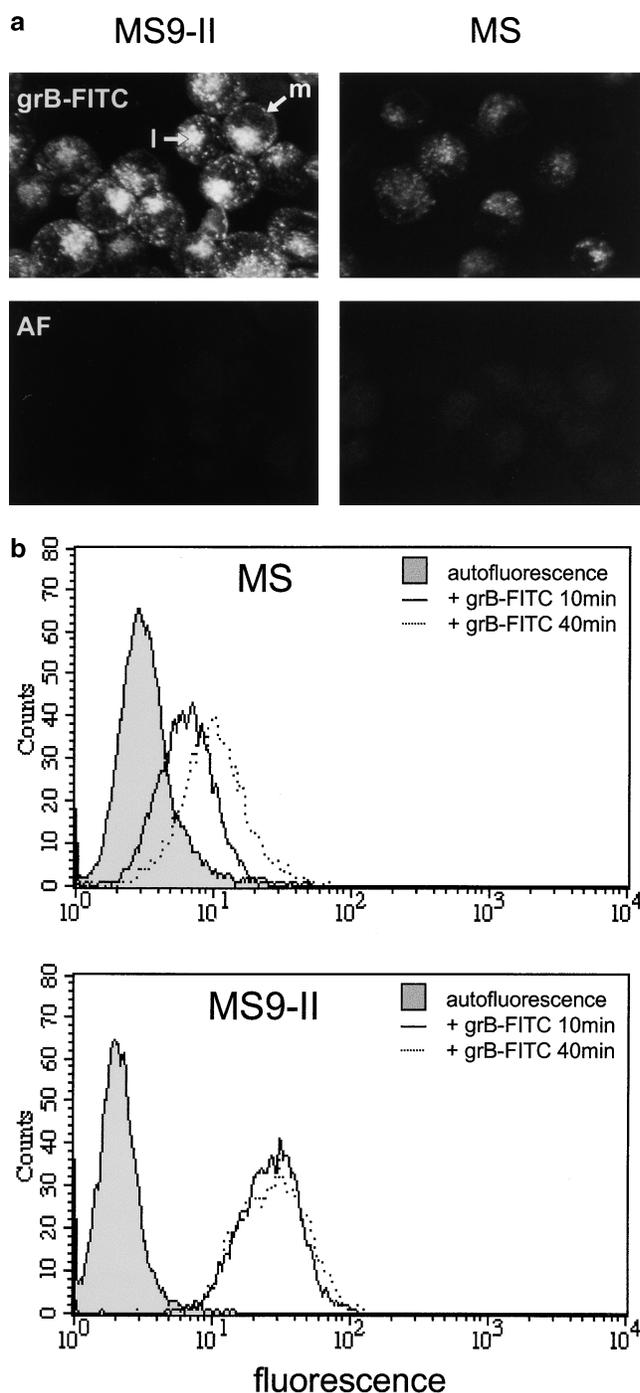
mit uptake into the cytoplasm. Consistent with the overexpression of MPR on the surface of MS9-II cells, FITC–granzyme B binding at the plasma membrane (expressed as the ratio of fluorescence at the plasma membrane to that in
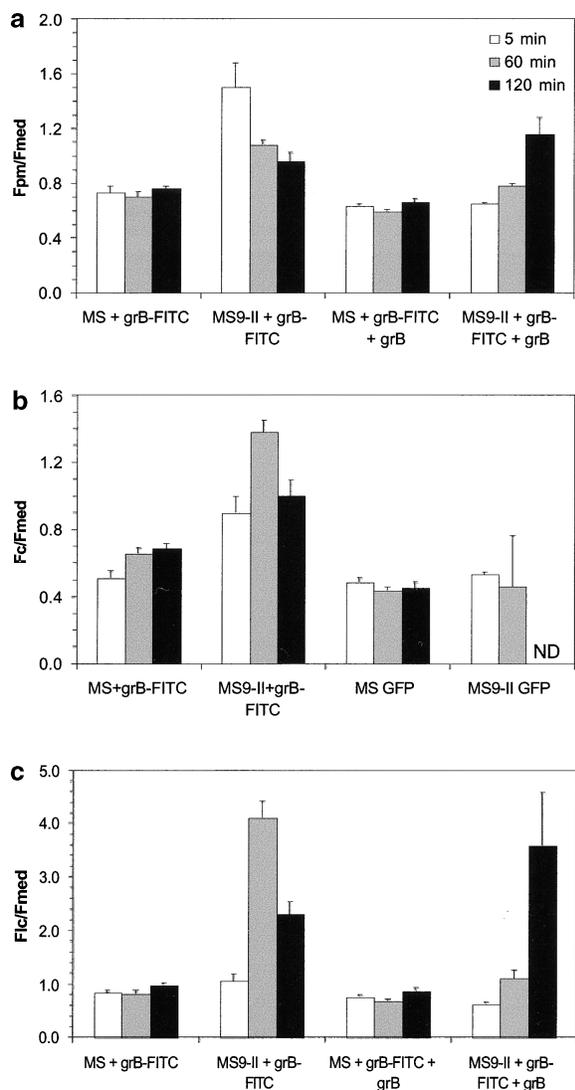
Figure 5. **Quantitative kinetic analysis of the uptake of FITC–granzyme B into MS and MS9-II cells.** Uptake of FITC–granzyme B (grB) or unglycosylated GFP by MS (MPR-null) and MS9-II (MPR-overexpressing) L cells. The uptake of both fluoresceinated molecules onto the plasma membrane (top) into the cell cytoplasm (middle) or into the late endosomal compartment (bottom) was quantitated by confocal laser scanning microscopy and image analysis as described previously (Jans, 1995). The results are expressed as a ratio between total cytoplasmic fluorescence (Fc), plasma membrane fluorescence (Fpm), or fluorescence of the late endosomal compartment (Flc) and extracellular fluorescence (Fmed) ± standard error of the mean. Each data point was derived from measurements on at least 30 cells selected at random after subtraction of autofluorescence (as described in Materials and methods).

B could block cell surface fluorescence of MS9-II but not MS cells indicated that although uptake of granzyme B into MS9-II cells was largely dependent on binding to MPR, the uptake into MS cells was not mediated by a cell surface receptor. Despite this, and consistent with findings presented above (Fig. 4), the cytoplasmic accumulation of FITC–granzyme B (Fc/Fmed) was not abolished in MS cells, although it was, as expected, reduced in comparison with MS9-II cells (Fig. 5 b). Significant and equivalent fluid phase uptake of unglycosylated GFP was seen into both cell lines, which did not vary appreciably over the 2 h of study. The trafficking of FITC–granzyme B to the late endosomal compartment (measured as the ratio of fluorescence of the late endosomal compartment and extracellular fluorescence, Flc/Fmed) was also much more rapid in MS9-II cells than MS cells and was markedly slowed by preaddition of unlabeled competitor, whereas trafficking to this compartment in MS cells was far less prominent and not influenced by competitor (Fig. 5 c).

Characterization of the kinetics of FITC–granzyme B entry into specific subcellular compartments of MS cells (Fig. 5, a–c) strongly suggested uptake through fluid phase endocytosis. First, MS cells showed no evidence of granzyme B binding to receptors on the plasma membrane that could be competed by unlabeled granzyme, whether at 4 or 37°C. Second, despite this, cytoplasmic accumulation of granzyme B was still observed in MS cells. Third, although the accumulation of FITC–granzyme B in the cytoplasm and the late endosomal compartment was significantly inhibited by unlabeled granzyme B in MS9-II cells, this was not the case in MS cells.

Our next aim was to compare the susceptibility of MS and MS9-II cells to granzyme B–mediated death. To try to maximize any difference between the two cell lines, they were exposed to limiting concentrations of granzyme B (3 and 12 nM) together with sublytic PLO for 1 h before quantitating cell death in clonogenic assays (Fig. 6 a). At these limiting granzyme B concentrations, the MPR-overexpressing cells were about twofold more susceptible to cell death, but the MPR-null MS cells were also sensitive, and there was no difference in colony formation when a slightly higher but conventional granzyme B concentration (50 nM) or higher concentrations (unpublished data) were used. To put the granzyme B concentrations we used in context, concentrations in the 1–5-μM range have been used by other investigators in similar assays (Thomas et al., 2000, 2001). The release of ^{51}Cr from MS cells after exposure to granzyme B and lytic agent was also reduced compared with MS9-II cells, but the difference between the two cell lines diminished with time (Fig. 6 b). After 1 h, the release of ^{51}Cr by MS cells was only 35% that of MS9-II cells, but by 6 h the release by MS cells had reached 60% of MS9-II.

The susceptibility of MPR-overexpressing and MPR-null L cells was also examined in response to intact allogenic CTL. Effector cells raised in both BALB/c (d anti-k) or C57BL/6 (b anti-k) mice induced the equivalent release of ^{51}Cr release from MS and MS9-II cells over a 4-h assay. However, MPR-null MS cells demonstrated significantly reduced DNA fragmentation (Fig. 7). DNA damage in both populations of L cells was virtually totally dependent on
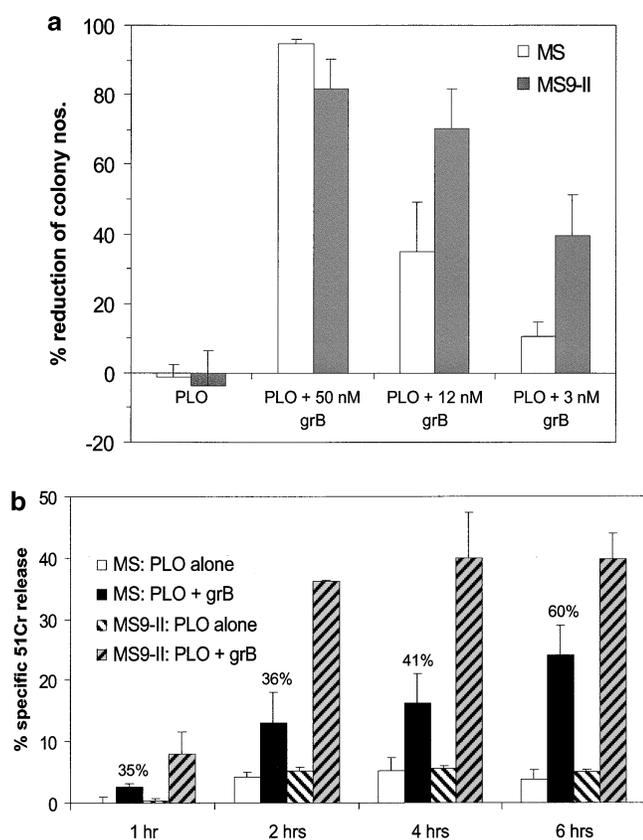
the extracellular medium, Fpm/Fmed) had reached maximal levels within 5 min and gradually diminished as the cells took up granzyme B into their cytoplasm (Fig. 5 a). Consistent with uptake through a cell surface receptor (MPR), preincubation with a 10-fold excess of unlabeled granzyme B virtually abolished plasma membrane fluorescence (Fig. 5 a, t = 5 min and later time points). By comparison, the level of plasma membrane staining of MS cells was much lower, did not vary over time, and was unaffected by the addition of unlabeled competitor. The finding that unlabeled granzyme

Figure 6. **MPR-null L cells are not protected from granzyme B–induced cell death.** (a) Clonogenic survival of MS and MS9-II cells after incubation with sublytic pneumolysin (PLO) alone or with various concentrations of granzyme B at 37°C for 90 min. Results are shown as the percentage inhibition of colony growth (mean of three cultures ± standard error at each granzyme concentration) compared with untreated cells. Typically, 75–150 L cell colonies grew when the cells were untreated. The experiment shown is representative of four similar experiments (as described in Materials and methods). (b) ^{51}Cr-labeled MS or MS9-II cells were incubated with sublytic pneumolysin (PLO) alone or together with granzyme B (50 nM) at 37°C for the times indicated. Specific ^{51}Cr release is shown as the mean of triplicate data points ± standard error. The assay is representative of three similar experiments.

Figure 7. **Reduced DNA fragmentation but not membrane permeability in MPR-deficient L cells killed by alloreactive CTL.** MS and MS9-II cells (H-2k) were prelabeled simultaneously with ^{51}Cr and ^{125}I-deoxyuridine, then incubated at 37°C for 4 h at the effector-to-target cell ratios indicated with alloreactive CTL raised in BALB/c (H-2d), C57BL/6, or C57BL/6.grB$^{-/-}$ (H-2b) mice (as described in Materials and methods). Specific release of ^{51}Cr (indicating cell membrane permeability) and ^{125}I-DNA (DNA fragmentation) were estimated as the mean of triplicate data points ± standard error. The experiment shown is representative of four similar experiments.

granzyme B, since granzyme B–deficient C57BL/6 CTL were unable to induce any release of ^{125}I-DNA from either cell line. Overall, our results were consistent with a role for the granzyme B-MPR pathway in facilitating DNA fragmentation rather than significantly influencing cell survival.

MPR is not required for allograft rejection

The previous report by Motyka et al. (2000) indicated that MS cells (H-2^k) implanted beneath the kidney capsule of BALB/c mice (H-2^d) were not rejected and grew to form tumors, whereas MPR-overexpressing MS9-II cells were rapidly rejected. This finding suggested a pivotal role for MPR in allotransplantation, with possible significant implications for the therapy of allograft rejection. Because of their potential importance, we decided to perform similar experiments with some additional controls. Consistent with the previous study, both MS and MS9-II cells formed rapidly growing tumors when implanted beneath the kidney capsule of im-
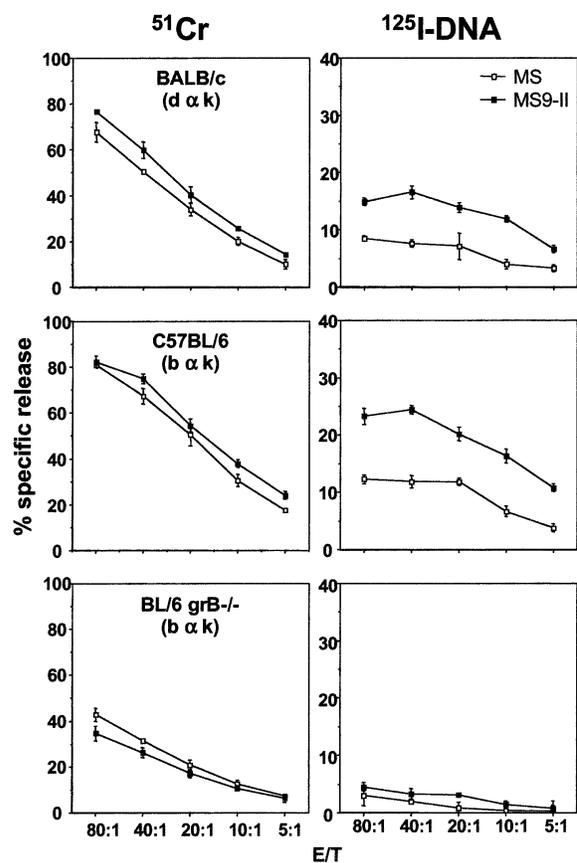
mune-compromised BALB/c.*scid/scid* mice. MS9-II tumors grew somewhat more rapidly and invaded the kidney parenchyma more readily than MS (Fig. 8, a and b). By contrast, both cell lines failed to form tumors in immunocompetent BALB/c mice. 7 d after implantation, small areas of MS9-II tumor could be identified histologically, invariably infiltrated with mononuclear cells, whereas MS tumor could not be identified (Fig. 8, c and d). By 14 d, both tumors were completely eradicated (Fig. 8, e and f). To determine the role of the granule (i.e., perforin/granzyme) pathway in this tumor rejection model, syngeneic perforin-deficient animals were inoculated with the same tumors. Surprisingly, both tumors were again rejected. Once more, transient tumor growth was seen in some animals at 7 d; however, complete rejection of both tumors occurred by day 14 (Fig. 8, g–j), leaving only dilated blood vessels, capsular thickening, and a patchy mononuclear cell infiltrate (Fig. 8 k). To assess tumor rejection in a different strain combination, we repeated the experiment in C57BL/6 (H-2^b) and granzyme B–deficient animals on the C57BL/6 background. Once again, both tumors were rejected (unpublished data). Collectively,

Figure 8.   **Growth of MS and MS9-II L cell tumors in immune-deficient BALB/c.*scid/scid* mice, but rejection by immunocompetent mice and mice lacking perforin expression.** MS or MS9-II cells were inoculated under the kidney capsule of groups of four recipients of the mouse strains indicated (as described in Materials and methods). Either 7 or 14 d later, the mice were killed and histological analysis of the kidneys was performed using hematoxalin and eosin staining of the formaldehyde-fixed tissues. Magnifications: (a–j) 200×; (k) 400×. BV, blood vessel; CT, connective tissue; L, mononuclear infiltrate.
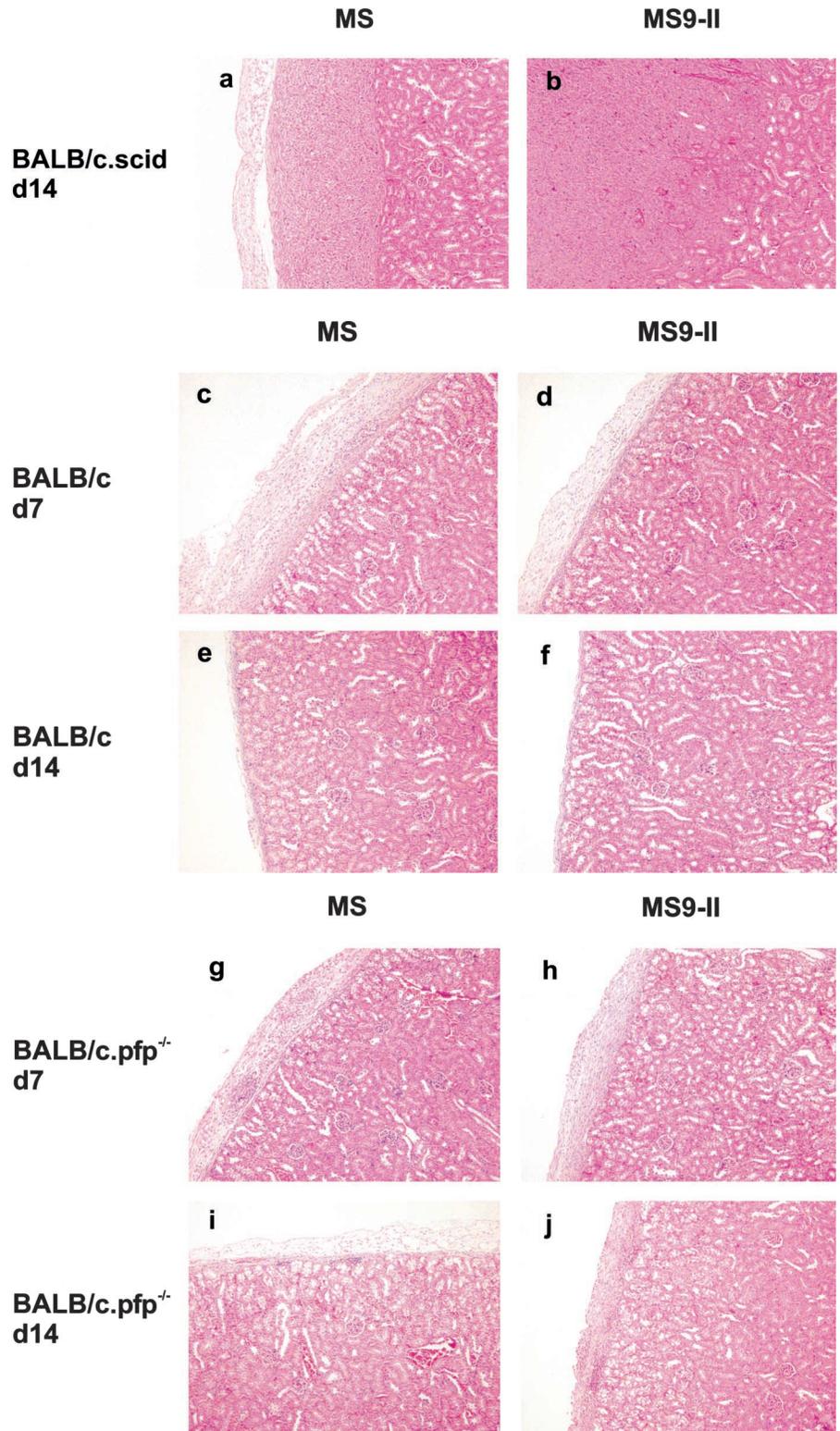
our findings clearly indicated that (a) MPR is not required for allograft rejection across a major histocompatibility mismatch and (b) rejection of both MS and MS9-II cells occurs independently of the granule pathway. In other experiments (unpublished data), we also found that Fas ligand mutant (gld) mice rejected MS and MS9-II tumors, indicating that cell death was not achieved through the Fas pathway.

Since rejection of both MS and MS9-II tumors by immunocompetent mice did not require either the perforin/granzyme or Fas pathways, we hypothesized that rejection in this model might be antibody mediated. Consistent with this hypothesis, when MS9-II cells were used to inoculate syngeneic C3H mice, the tumors also failed to grow and the mice developed high titer antibodies that reacted with MS9-II cells

MS

Discussion

and at lower but significant levels with MS cells. Typically, the antibody titers by indirect immunofluorescence were ~1/1,600 on MS9-II cells and 1/200 on MS cells (unpublished data). We concluded that human MPR expressed on MS9-II cells is a target of the anti–MS9-II antibody response. As a result of long term culture in vitro, it is also likely that MS and MS9-II express other immunogenic antigens responsible for perforin/granzyme B–independent rejection of MS cells. To definitively demonstrate a role for antibody in tumor rejection in vivo, we adoptively transferred serum from C3H mice that had been challenged twice with MS9-II cells into four BALB/c.*scid.scid* mice 1 d before, and on the same day as they were implanted with MS9-II cells under the kidney capsule. Control mice received serum from unimmunized C3H mice. 7 d later, tumor growth was clearly evident in four out of four control mice; however, three out of four mice pretreated with anti–MS9-II antiserum showed complete absence of tumor, whereas the fourth mouse showed significantly reduced tumor mass (representative data in Fig. 9). These results clearly indicated that rejection of MS9-II was antibody mediated in immunocompetent animals and related to expression of immunogenic (human) MPR by these cells. Therefore, MPR played no role in cell-mediated tumor allograft rejection in these or the previously reported studies.

It is known that granzyme B is able to bind to MPR, both in the context of intracellular trafficking in a CL in which it is synthesized (Griffiths and Isaaz, 1993) and when MPR is expressed on the surface of a target cell (Motyka et al., 2000). Since overexpression of MPR on the cell surface enables a cell to take up greater quantities of granzyme B through endocytosis, it is not surprising that such a cell should be a better target for the granzyme B death pathway in concert with perforin (Motyka et al., 2000; this study). However, no study has previously determined whether susceptibility to granzyme B–induced death is lost if MPR is absent or uptake through it is abolished. The significance of the present study is that it sought to determine whether cells in which granzyme B cannot be taken up through the MPR remain susceptible to granzyme B–mediated cell death either in vitro or in vivo. Our findings clearly indicate that human HeLa cells and mouse L cells possess more than one pathway for taking up granzyme B into their cytoplasm. One pathway utilizes granzyme B binding to MPR as described previously. However, MPR binding is not critical to granzyme B–induced cell death, since constitutive nonreceptor-mediated fluid phase endocytosis effectively delivers proapoptotic granzyme B independently of MPR. Consistent with "bulk"
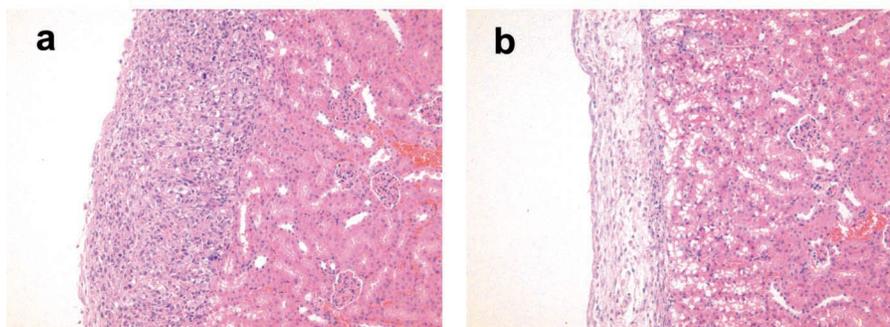

Figure 9. **Adoptive transfer of anti–MS9-II antiserum mediates graft rejection.** MS9-II cells were inoculated under the kidney capsule of groups of four BALB/c.*scid.scid* recipients (as described in Materials and methods). The groups received nonimmune C3H serum (a) or anti–MS9-II antiserum as described in Materials and methods. Magnification, 200×.

uptake of granzyme B, this second pathway is characterized by a lack of appreciable granzyme B binding to the plasma membrane and lack of inhibition of cytoplasmic uptake by unlabeled competitor granzyme. It is known that fluid phase endocytosis remains active in K44A-dynamin–expressing cells, and indeed, HeLa cells can survive near total blockade of the clathrin/dynamin pathway through the constitutively active fluid phase mechanism, enabling the uptake of essential nutrients (Damke et al., 1995).

Although virtually all receptor-mediated uptake is thought to be blocked in K44A-dynamin–expressing cells, the defect in uptake in MS cells is restricted to MPR. This difference between our two models is significant for two reasons. First, since ligand/receptor complexes other than MPR and its cargo can be taken up normally into MS cells, the fluid phase uptake of granzyme B cannot be considered an aberration seen only when the clathrin/dynamin-mediated uptake is completely nonfunctional. This means that both receptor-mediated and receptor-independent uptake of granzyme B can coexist in the same cell. Second, the fact that fluid phase uptake was observed in MPR-deficient MS cells suggests that no other cell surface receptor capable of binding granzyme B with physiologically relevant avidity is expressed by these cells. We cannot totally exclude the possibility that cell surface receptors other than MPR exist for granzyme B; however, their absence from L cells indicates that, unlike MPR, they are not expressed in every cell type.

Perturbation of MPR expression has been causally associated with certain malignancies, particular with hepatocellular dysplasia and carcinoma. Loss of heterozygosity at the MPR locus is relatively common in these tumors as are mutations in the remaining allele. Thus, MPR can function as a tumor suppressor gene in some tissues (Morita et al., 1991; De Souza et al., 1995; Chappell et al., 1997). Given the recent finding that MPR can bind granzyme B (Motyka et al., 2000; this study), it is tempting to interpret the loss of MPR function as a means of tumor escape from CL attack, particularly as CL are important in defense against hepatitis viruses that frequently predispose to malignant transformation. Such speculation must as yet be tempered by the observation that MPR plays many important functions other than binding granzyme B. MPR binds to several ligands that can affect cell proliferation and differentiation, including insulin-like growth factor II (Oka and Czech, 1986), the precursor form of transforming growth factor β (Kovacina et al., 1989), and leukemia inhibitory factor (Blanchard et al., 1998), that might equally influence malignant transformation through alternative mechanisms.

Materials and methods

Mice
BALB/c, BALB/c.scid/scid, BALB/c.Pfp$^{-/-}$ (perforin-deficient), C57BL/6, C57BL/6.gzmB$^{-/-}$ (granzyme B-deficient), and C3H mice were purchased from the Walter and Eliza Hall Animal Facility (Parkville, Australia) or bred at the Peter MacCallum Cancer Institute Animal Facility. The Peter MacCallum Cancer Institute Animal Welfare Committee approved the animal studies described herein.

Chemicals and reagents
Human perforin was purified from the natural killer cell line, YT, as described (Sutton et al., 1997). PLO was obtained from Dr. James Paton (Women's and Children's Hospital, Adelaide, South Australia) and activated in PBS-containing β-mercapto-ethanol. A sublytic dose of perforin or PLO was defined as producing <10% specific release of ^{51}Cr in a 4-h assay at 37°C and was determined independently for each cell line. Native human granzyme B was immunopurified from YT cell lysates as described (Trapani et al., 1993). The granzyme B was free of other granzyme activities and perforin as demonstrated by Western blotting and proteolytic assays, respectively. Purified granzyme B and human transferrin (purchased from Sigma-Aldrich) were labeled with FITC as described (Trapani et al., 1996) and stored at 4°C until use. Polyclonal antiserum detecting MPR was a gift from Dr. William Sly (Washington University, St. Louis, MO). The monosodium salts of G6P and M6P were purchased from Sigma-Aldrich, dissolved in PBS at 200 mM, and stored at –20°C.

Cell lines
HeLa cells stably overexpressing either mutated (K44A) or wild-type dynamin under the control of a tet-sensitive promoter were obtained from Dr. Sandra L. Schmid (The Scripps Research Institute, La Jolla, CA). The cells were maintained in DME medium containing 10% FBS, G418 (400 μg/ml), puromycin (200 μg/ml), L-glutamine (2 mM), and tet (1 μg/ml). To induce expression of dynamin, the cells were harvested, washed, and resuspended in medium lacking tet for 24 h at 37°C. The cells were then grown in fresh tet-free medium for a further 24 h before use. The mouse L cell line MS9-II (H-2^k) derived by overexpressing the human MPR in MPR-null L cells was obtained by permission of Dr. William Sly (Washington University, St. Louis, MO), from Dr. Chris Bleackley (University of Alberta, Alberta, Canada) (Motyka et al., 2000). Parental MPR-deficient MS cells transfected with vector DNA alone were used as a control in all experiments (Motyka et al., 2000).

Cell death assays
Allogenic (anti-H-2^k) CTL were raised by immunizing BALB/c (d anti-k) or C57BL/6 (b anti-k) mice with freshly isolated C3H splenocytes (5 × 10^5) injected into each hind footpad in PBS (50 μL). The immunization was repeated 2 wk later. 4 d after the second immunization, the mice were killed and the popliteal lymph nodes were isolated, teased into a single cell suspension, plated at 5 × 10^6 cells/ml in RPMI medium, and cultured at 37°C for 3 d in a humidified CO$_2$ incubator. The cells were then harvested and used in cytotoxicity assays. The specific release of ^{51}Cr, a measure of plasma membrane permeability, and ^{125}I-DNA from target cells, a measure of DNA fragmentation, were determined as described (Sutton et al., 1997). For clonogenic survival assays, HeLa cell transfectants were plated in triplicate at ~150 cells/well in the presence or absence of tet into a 24-well plate. Cells were incubated with perforin (or PLO in some experiments) in the presence or absence of granzyme B (3–50 nM) for 2 h at 37°C. The wells were then flooded with medium containing 10% (vol/vol) FBS and incubated for a further 3–5 d at 37°C when discrete colonies were counted. Similar colony-forming assays were also performed with MS and MS9-II cells.

Confocal microscopy and FACS® analysis
HeLa cells grown in the presence or absence of tet were washed in Hank's buffered salt solution (HBSS), incubated with FITC–granzyme B or FITC-transferrin for 10–45 min at 37°C, then washed, fixed in 2% PFA, and centrifuged onto glass slides before confocal microscopy. In some experiments, cells were preincubated in HBSS containing M6P or G6P (5 mM) for 15 min before adding FITC–granzyme B or FITC-transferrin and analysis by flow cytometry. For quantitative and kinetic studies of granzyme B uptake, cells were exposed to FITC–granzyme B for various times at 37°C, and uptake onto the plasma membrane or into the cell cytoplasm was evaluated as described previously (Jans, 1995). Image analysis on digitized confocal images was performed using the Macintosh NIH Image 1.49 public domain software.

Transplantation experiments
Sterile MS or MS9-II L cell fibroblasts (certified Mycoplasma free) were harvested in logarithmic growth phase, washed several times in PBS, and resuspended at 100 million cells/ml in PBS. Mice (groups of four of each strain) were deeply anaesthetised with methoxyflurane, placed in the supine position under a heat lamp, and a midline incision was made through the skin, fascia, and peritoneum. Each kidney, in turn, was identified and a small incision was made in the kidney capsule, close to its caudal pole. Under a dissecting microscope, a flexible plastic cannula was introduced through the incision into the subcapsular space and advanced carefully toward its cephalad pole. Cells (2 million in 20 μL PBS) were then deposited into the subcapsular space, and the cannula slowly withdrawn. The skin and peritoneum were repaired, and the animals were allowed to recover

from anesthesia under the heat lamp for ∼1 h. After 7 or 14 d, the animals were killed by cervical dislocation, and the kidneys were recovered and fixed immediately in formaldehyde for sectioning, hematoxalin/eosin staining, and histological analysis. In some experiments, mice were bled after various time intervals to quantify antibody responses to the tumors.

In another experiment, eight C3H mice were injected subcutaneously with 5 million live MS-9 cells (without adjuvant) on two occasions, 14 d apart. 10 d later, the mice were killed, and their total blood volume was immediately harvested by cardiac puncture under direct vision. Serum was prepared from the pooled blood and from the same number of unimmunized C3H mice and stored at –80°C until required. Groups of 4 BALB/c.scid mice were inoculated with 1.0 ml of serum from either the immunized or unimmunized mice into the peritoneal cavity 1 d before and on the same day as they were injected with MS-9 cells under the kidney capsule, exactly as described above. The mice were killed 7 d later, and their kidneys were recovered, fixed, and examined histologically as described above.

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