Restoration of a Tumorigenic Phenotype by β_2-Microglobulin Transfection to EL-4 Mutant Cells

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Summary

It has frequently been suggested that loss of β_2-microglobulin (β_2m) in tumor cells may lead to malignant progression due to escape from immunological recognition. Here, we directly tested the role of β_2m expression in tumorigenicity. A β_2m loss mutant (C4.4-25$^-$), selected from the murine lymphoma EL-4, showed a marked reduction in tumorigenicity as compared with EL-4 in normal C57Bl/6 (B6) mice. The reduced tumorigenicity was directly related to β_2m expression. Transfection of an intact murine β_2m gene markedly increased the tumorigenic potential. The reduced tumorigenicity of C4.4-25$^-$ compared with β_2m transfected cells was observed also in athymic B6 *nu/nu* mice, but was abolished in B6 mice depleted of natural killer (NK) 1.1–positive cells. These results show that restoration of β_2m expression can promote tumorigenicity and demonstrate for the first time that induction of major histocompatibility complex class I expression by transfection can lead to escape from NK cells in vivo.

MHC class I expression is often reduced or completely lost in tumors (1). These molecules serve as restriction elements for CTL by presenting antigenic peptides derived from intracellularly degraded proteins (2). Thus, it has frequently been suggested that downregulated class I expression may contribute to tumor progression by allowing escape from CTL-mediated lysis. Loss of β_2-microglobulin (β_2m)[1] is one cause of MHC class I deficiency in tumors. In one study of 15 MHC class I–deficient colorectal adenocarcinomas, loss of β_2m was responsible for the absence of mature HLA-A, -B, and -C heavy chains in all 15 cases (3). Accordingly, loss of β_2m has been predicted to promote malignancy, although this has never been tested formally.

To experimentally address the role of β_2m in malignancy is not trivial, since target MHC class I molecules appear to have a protective effect against recognition by NK cells (4). This concept predicts an opposite effect of β_2m loss in tumorigenicity. Loss of β_2m should reduce tumorigenicity due to elimination by NK cells while re-expression should restore it.

We set out to test the role of β_2m in tumorigenicity in a murine mutant-transfection model. A β_2m-deficient mutant of the highly tumorigenic lymphoma line EL-4 was selected and subsequently transfected with a genomic clone of the murine β_2m^b gene. Transfections resulted in class I–expressing cells which were used together with the β_2m-deficient mutant to test tumorigenicity in syngeneic B6 and corresponding athymic B6 *nu/nu* mice, as well as in B6 (C57Bl/6) mice depleted of NK-1.1-positive cells. The outcome of the results was the opposite of that predicted from CTL studies. Loss of β_2m expression was associated with reduced tumorigenicity, and restoration of β_2m expression led to an increased tumorigenic potential.

Materials and Methods

Selection of Mutant Cell Lines and DNA-mediated Gene Transfection. For selection of the β_2m-deficient mutant C4.4-25$^-$, parental EL-4 (H-2^b) lymphoma cells were treated twice with 10 μg/ml MNNG (*N*-methyl-*N'*-nitrosoguanidin). Surviving cells were subjected to complement treatment (Low Tox Rabbit Complement; Cedarlane, Labs., Ltd., Hornby, Ontario, Canada) after incubation with a mixture of anti-H-2K^b and D^b mAbs B22.249, K10-56, and 28-8-6S; mAbs (5). After recovery of surviving cells, the complement treatment was repeated three times. Cells were then subcloned four times by limiting dilution or single-cell sorting on a cell sorter (Coulter Corp., Hialeah, FL). Transfection was performed with a genomic β_2m^b clone combined on the same plasmid (constructed and kindly provided by Dr. P. Robinson, Medical Research Council, Harrow, UK) as the neo^R gene by electroporation, as described (5). Selection after transfection was carried out in medium with 1.0 mg/ml or geniticin (G418, Gibco Laboratories, Uxbridge, UK). In vitro carried mutant and transfectant lines

[1] *Abbreviations used in this paper:* β_2m, β_2-microglobulin; B6, C57Bl/6; MNNG, *N*-methyl-*N'*-nitrosoguanidin.

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showed no detectable difference in growth rate in normal tissue culture medium.

Source of Mice, In Vivo Tumorigenicity Tests, and NK Cell Depletion. B6 and BALB/c mice were bred and maintained at the Department of Tumor Biology, Karolinska Institutet, or purchased from ALAB, Sollentuna, Sweden. Athymic B6 *nu/nu* mice were purchased from Gl. Bomholtgard, Ry, Denmark. Mice used in in vivo experiments were 5–8 wk old at the start of the experiments, usually littermates, or otherwise age-matched within 2 wk. Graded doses of in vivo-passaged (6) tumor cells were inoculated either subcutaneously (right flank) or intraperitoneally in a 0.1 ml vol of PBS. Tumors always appeared at the site of inoculation, and tumor growth was followed at least once weekly by palpations and measurements at the tumor site. Mice were killed when the subcutaneous tumors reached a size of >15 mm in diameter, and no signs of rejection were observed. Mice without any signs of tumor growth were kept under observation for at least 30 d after inoculation. Small groups of mice, never exceeding five mice per group, were tested in several independent tests throughout the study to minimize the risk of random fluctuations in the quantity or quality of cells inoculated. For NK cell depletion in vivo, mice were given a single injection of 200 μl ascites fluid prepared from mice inoculated intraperitoneally with the anti-NK1.1 mAb 1 d before inoculation of tumor cells (7).

Immunoprecipitation and SDS-PAGE. Immunoprecipitation of MHC class I molecules from [^{35}S]methionine metabolically labeled cells with a rabbit anti-mouse class I antiserum (K270, kindly provided by Dr. L. Rask, Biomedical Center, Uppsala, Sweden) and subsequent analysis on a 13% SDS-PAGE was carried out as described (8).

FACS® Analysis. The cell surface expression of H-2D^b and K^b shown in Table 1 was measured on a FACS IV® (Becton Dickinson & Co., Mountain View, CA) with the anti-H-2D^b (28-14-8S) anti-H-2K^b (28-13-3S) mAbs from the American Type Culture Collection (Rockville, MD).

In Vitro Cell-mediated Cytotoxicity. Anti-H-2^b–specific CTL effectors were generated in bulk MLCs by using splenocytes from BALB/c mice, preimmunized with B6 splenocytes, as responders and irradiated splenocytes from B6 mice as stimulators. CTL were H-2^b specific since they also killed other H-2^b-expressing targets (RBL-5), but not H-2^d–expressing targets (P815) (data not shown). A standard 4-h ^{51}Cr-release assay was used to measure cytotoxic activity.

Results and Discussion

FACS® analysis demonstrated that the EL-4-derived C4.4-25$^-$ line was class I deficient on the cell surface (Table 1). This can be explained by lack of β_2m expression. No β_2m could be coprecipitated with the class I H chains as revealed by immunoprecipitation and subsequent SDS-PAGE analysis (Fig. 1). Note the total absence of β_2m and reduced molecular weight of the class I H chains isolated from the C4.4-25$^-$ mutant (Fig. 1), the latter due to an arrest in transport through the Golgi complex where terminal glycosylation (increasing the mol wt) of class I H chains normally would occur (9). The mutant was completely resistant to conventional anti-H-2^b allo-specific CTL lysis (Table 1). C4.4-25$^-$ was transfected with a genomic clone of the murine β_2m^b gene. Two transfected clones, E50.15$^+$ and E50.16$^+$, were isolated for further characterization. These clones both expressed

Table 1. *Cell Surface MHC Class I Expression and CTL Sensitivity of EL-4 Wild-Type, Mutant, and Transfectant Cell Lines*

			EL-4	C4.4-25$^-$	E50.16$^+$
Cell surface class I expression*	H-2D^b		158	4	138
	H-2K^b		166	2	141
CTL sensitivity‡	E/T	40:1	91	0	81
		10:1	83	0	67
		2.5:1	69	0	39
		0.6:1	44	0	20
		0.16:1	21	0	9

* Mean linear fluorescence value (background subtracted), one representative analysis shown.
‡ Percent specific lysis, one representative experiment shown.

β_2m which could be coprecipitated with the class I H chains (Fig. 1; E50.15$^+$, data not shown). They expressed class I molecules on the cell surface (Table 1; the E50.16$^+$ line expressed 62% [SD 27%] of D^b and 65% [SD 25%] of K^b compared with the EL-4 wild type line in an average of three independent experiments) and could be killed by allo anti-H-2^b-specific CTL (Table 1; E50.15$^+$, data not shown).

Graded tumor doses of EL-4, C4.4-25$^-$, and E50.16$^+$ cells were inoculated subcutaneously or intraperitoneally in normal

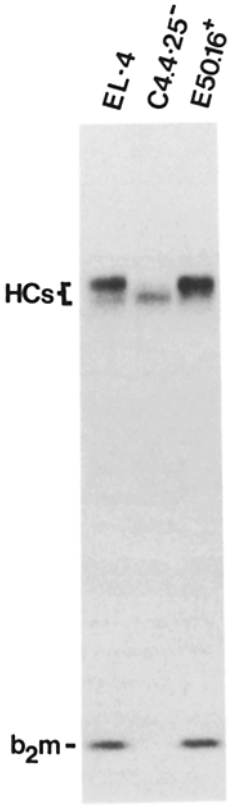

Figure 1. SDS-PAGE analysis of class I molecules immunoprecipitated from [^{35}S]methionine-labeled EL-4 wild-type, mutant, and transfectant cells. Immunoprecipitates made with a rabbit anti-mouse class I antiserum. HCs, heavy chains.

B6 mice, and tumor growth was followed. The C4.4-25$^-$ β_2m–deficient mutant line showed a markedly reduced tumorigenic potential compared with the EL-4 wild-type line (Table 2). In contrast, the β_2m-transfected C4.4-25$^-$ line, E50.16$^+$, had regained tumorigenicity when compared with C4.4-25$^-$. When tumors appeared after inoculation of C4.4-25$^-$ mutant cells (Table 2), the latency period was usually 2–3 wk longer than tumors derived from inoculates of E50.16$^+$ cells. The effect of the β_2m gene was not unique for the E50.16$^+$ transfectant. The E50.15$^+$ β_2m–transfected cell line also formed solid tumors after subcutaneous inoculation of 10^4 cells in four of five mice, and C4.4-25$^-$ did not form any tumors in five mice inoculated in the same experiment.

The differences in tumorigenicity between C4.4-25$^-$ and E50.16$^+$ transfectant lines remained in athymic nude (*nu/nu*) mice (Table 2). However, the tumorigenicity of C4.4-25$^-$ compared with E50.16$^+$ was restored in mice depleted of NK1.1-positive cells. In these mice both lines grew equally well (Table 2). This suggested that the C4.4-25$^-$ line was eliminated by NK cells in vivo. Supporting this notion was an enhanced NK sensitivity in vitro of the C4.4-25$^-$ line compared with EL-4 and E50.16$^+$ (data not shown). Also compare the enhanced NK sensitivity in vitro of other β_2m-deficient EL-4 lines (5).

It should be noted that the tumorigenic potential of the E50.16$^+$ transfectant never reached that of the EL-4 wild-type line although it was significantly increased compared with the C4.4-25$^-$ line (Table 2). This result, at least partly, may be a reflection of the small (but consistent) reduction of class I levels on E50.16$^+$ compared with the EL-4 wild-type line (Table 1). E50.16$^+$ was also slightly less killed by CTL than EL-4 (Table 1). Note that the E50.16$^+$ line was somewhat more tumorigenic in NK1.1-depleted mice than in normal untreated B6 mice (Table 2), which is consistent with a significantly reduced, but not totally abrogated NK-sensitive phenotype in vivo.

To our knowledge, this is the first time that the β_2m gene directly has been demonstrated to protect a tumor from rejection and thus promote tumorigenicity. The results suggest that the effect of β_2m is due to altered interactions with the host, not due to the intrinsic growth properties. We conclude that NK cells eliminate the C4.4-25$^-$ cells because of the MHC class I–deficient phenotype and that this elimination is abrogated by restoration of MHC class I expression at the cell surface. The results fit with in vitro results, demonstrating that NK cells can recognize and kill cells that fail to express MHC class I molecules, but not similar cells after restoration of MHC class I expression by class I/β_2m transfection (reviewed in reference 4). They also support earlier studies in which MHC class I–deficient lymphoma cells were shown to be eliminated by NK cells in vivo (6, 10). The present results are in line with the observation that expression of recipient MHC class I molecules can protect tumor or bone marrow grafts from rapid elimination by NK cells. Expression of a H-2D^d transgene protects B6 bone marrow, as well as lymphoma cells from rejection in H-2D^d transgenic B6 mice (11–13). Furthermore, β_2m-deficient bone marrow cells are rejected by β_2m-expressing mice, and the marrow from β_2m +/− littermates is accepted (14; Öhlén et al., manuscript submitted for publication).

The present results differ from several earlier studies of

Table 2. *Tumor Growth after Subcutaneous or Intraperitoneal Inoculation of EL-4 Wild-Type, Mutant, and Transfectant Cell Lines*

Mice (pretreatment)	Inoculation site	No. of Cells inoculated	Cell lines		
			EL-4	C4.4-25$^-$	E50.16$^+$
C57Bl/6	Subcutaneous	10^3	18/20*	0/15	1/20
C57Bl/6	Subcutaneous	10^4	20/20	1/20	17/29‡
C57Bl/6	Subcutaneous	10^5	20/20	2/20	28/35‡
C57Bl/6	Intraperitoneal	10^3	13/13	4/18	9/13‖
C57Bl/6	Intraperitoneal	10^4	14/14	6/19	12/14§
C57Bl/6	Intraperitoneal	10^5	14/14	11/19	14/14‖
C57Bl/6 *nu/nu*	Subcutaneous	10^5	15/15	1/12	13/14‡
C57Bl/6 (NK 1.1)	Subcutaneous	10^3	ND	10/10	8/11
C57Bl/6 (NK 1.1)	Subcutaneous	10^4	ND	10/10	10/10
C57Bl/6 (NK 1.1)	Subcutaneous	10^5	ND	10/10	10/10

* Number of mice with tumor growth/total number of mice inoculated; in five cases regression of established solid tumors was seen after subcutaneous inoculation of E50.16$^+$ cells (one mouse inoculated with 10^3 cells, four mice inoculated with 10^5 cells).
‡ $p < 0.001$ by χ^2 analysis when compared with corresponding dose of C4.4-25$^-$ cells.
§ $p < 0.01$.
‖ $p < 0.05$.

MHC-dependent tumor rejection, many of which were performed with highly antigenic tumors, where transfection of class I molecules resulted in loss of tumorigenicity through escape from T cell immunity (reviewed in reference 15). Tumorigenicity can also be altered by MHC class I transfection through mechanisms independent of the host immune response (16, 17). Our results demonstrate a third possibility: reduced tumorigenicity associated with loss of class I expression due to NK cell–mediated rejection.

This does not permit the generalized conclusion that β_2m is a malignancy-promoting gene. The effects of variations in class I expression will depend on the dominating immunosurveillance system in a certain situation, i.e., NK- (eliminating class I–deficient cells) or T cells (restricted to elimination of class I–positive cells if these harbor antigenic peptides bound to the class I antigen-binding groove) (discussed in reference 4). However, the present study demonstrates that restoration of β_2m/class I expression, under certain circumstances, can lead to increased tumorigenicity.

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