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Antitumor effect of allogenic fibroblasts engineered to express Fas ligand (FasL)

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Fas ligand is a type II transmembrane protein which can induce apoptosis in Fas-expressing cells. Recent reports indicate that expression of FasL in transplanted cells may cause graft rejection and, on the other hand, tumor cells may lose their tumorigenicity when they are engineered to express FasL. These effects could be related to recruitment of neutrophils by FasL with activation of their cytotoxic machinery. In this study we investigated the antitumor effect of allogenic fibroblasts engineered to express FasL. Fibroblasts engineered to express FasL (PA317/FasL) did not exert toxic effects on transformed liver cell line (BNL) or colon cancer cell line (CT26) in vitro, but they could abrogate their tumorigenicity in vivo. Histological examination of the site of implantation of BNL cells mixed with PA317/FasL revealed massive infiltration of polymorphonuclear neutrophils and mononuclear cells. A specific immune protective effect was observed in animals primed with a mixture of BNL or CT26 and PA317/FasL cells. Rechallenge with tumor cells 14 or 100 days after priming resulted in protection of 100 or 50% of animals, respectively. This protective effect was due to $CD8^+$ cells since depletion of $CD8^+$ led to tumor formation. In addition, treatment of pre-established BNL tumors with a subcutaneous injection of BNL and PA317/FasL cell mixture at a distant site caused significant inhibition of tumor growth. These data demonstrate that allogenic cells engineered with FasL are able to abolish tumor growth and induce specific protective immunity when they are mixed with neoplastic cells.

Keywords: *Fas ligand; tumor; gene transfer; animal*

Introduction

Fas ligand (FasL, CD95L, APO-1L) is a type II transmembrane protein of the tumor necrosis factor family that induces apoptosis in Fas-positive cells.[1–3] It is predominantly expressed in activated T cells and it is one of the effector molecules for cytotoxic T lymphocyte (CTL) and NK cell-induced apoptosis.[4–6] Mice and humans, with a defect in Fas expression or function demonstrate a profound lymphoaccumulative disorder together with age-associated autoimmune phenomena.[7–9] Mutation of FasL gene in mice resulted in similar phenotype (gld-generalized lymphoproliferative disease).[10] This suggests that the Fas/FasL system plays an important role in the maintenance of immune homeostasis.[11,12]

Sertoli cells in the testis and epithelial cells in the anterior chamber of the eye also express FasL.[13–15] The presence of FasL confers immunological privilege to these cells and allo- or xenografts transplanted into these sites are not rejected since FasL expressed by Sertoli cells and epithelial cells of the anterior chamber of the eye induce death of infiltrating T cells expressing high levels of Fas.[15] Lau and co-workers[16] reported that syngenic myoblasts engineered to express FasL could delay rejection of pancreatic islet allografts when transplanted under the kidney capsule. Recently, expression of FasL was found on some tumor cells such as melanoma, colon cancer cells or hepatocellular carcinoma cells and these FasL-expressing tumor cells were capable of inducing apoptosis of lymphocytes, suggesting that this might be an additional mechanism for malignant cells to escape immunosurveillance.[17–20]

On the other hand, it was shown that the presence of FasL can involve destruction of FasL-bearing cells. First, Seino *et al*[21] showed that introduction of FasL cDNA into murine tumor cells did not affect growth *in vitro* but caused rejection *in vivo*. Further, Kang *et al*[22] reported that transplantation of islets of Langerhans infected with an adenoviral vector containing FasL into allogenic diabetic hosts underwent accelerated rejection. Transgenic expression of CD95 ligand on islet β cells induced a granulocytic infiltration but did not confer immune privilege upon islet allografts.[23] The authors demonstrated that this type of rejection was T cell and B cell independent and was mediated mainly by neutrophils, which involved local inflammation with further destruction of neighboring tissues. Until now it has not been known why the presence of FasL on the surface of cells can confer a state of immune privilege in some settings while in others it may precipitate inflammation and cell destruction.[24,25] The current study was undertaken to analyze whether allogenic fibroblasts engineered to express FasL could abrogate tumorigenicity and induce specific immune response to tumor antigens in a murine animal model.

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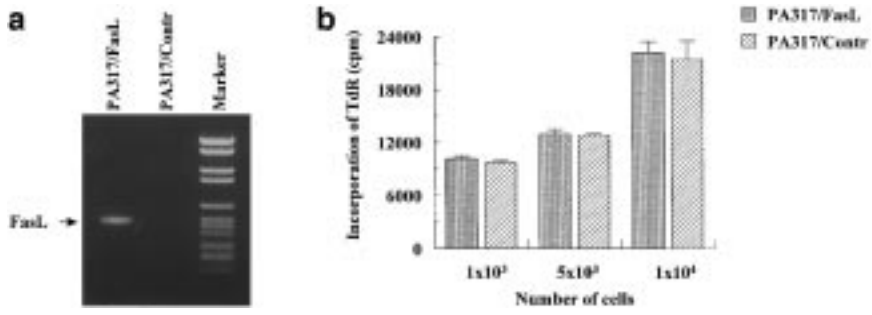

Figure 1 *Stable transfectants of fibroblast cell line PA317 were obtained by transfection of the cells with either pCl/FasL or control plasmid pCl and selection in geneticin-containing medium for 2 weeks. (a) RT-PCR using FasL-specific primers of one colony transfected with pCl/FasL expressing high level of FasL (PA317/FasL) and one colony transfected with control plasmid pCl which did not express FasL (PA317/Contr). 502 bp of FasL fragment was only seen in PA317/FasL cells after PCR product was run at 1.5% of agarose gel. (b) The two cell colonies PA317/FasL and PA317/Contr exhibit similar growth rate as estimated by incorporation of ^{3}H-TdR at different cell densities. Each bar represents mean ± s.d. of triplicate determinations.*

Results

Stable transfectants of fibroblasts expressing functional FasL

Stable transfectants of fibroblasts were produced by transfection of PA317 cells with pCl/FasL or pClneo. Transfected cells were selected in geneticin-containing medium and 10 colonies from both transfections were obtained for screening of FasL expression. Among these colonies, we selected one colony from pCl/FasL transfection which expressed a high level of FasL (PA317/FasL) and one colony from pClneo transfection (lacking FasL expression) was taken as a control (PA317/Contr) (see Figure 1a). As shown in Figure 1b growth rate (as estimated by incorporation of ^{3}H-TdR) was similar for PA317/FasL and PA317/Contr at different densities of cells.

To assess the function of FasL in transfected cells, we incubated PA317/FasL or PA317/Contr with YAC-1 cells. These cells are Fas positive and have been used in other studies as target cells for Fas/FasL interaction.[16] As shown in Figure 2b, PA317/FasL killed YAC-1 cells in a dose-dependent manner. We observed 31.2, 54.1, 58.9 and 69.3% YAC-1 cell death when effector cells were cocultured with target cells at effector/target ratios of 0.2:1, 0.6:1, 2:1 and 6:1, respectively. In contrast, PA317/Contr did not induce apoptosis at any effector/target ratio tested. We also investigated whether supernatants from PA317/FasL or PA317/Contr cells caused toxicity on YAC-1 cells. Figure 2c shows that 35, 25 and 20% of cell death occurred when YAC-1 cells were cultured with supernatants from PA317/FasL at 1:1, 1:5 and 1:10 dilution, respectively, while no toxic effect on YAC-1 cells was found when using supernatant from PA317/Contr cells. Typical apoptotic DNA ladder was seen when YAC-1 cells were co-incubated with PA317/FasL cells, but not with PA317/Contr cells (Figure 2a). These data indicate that PA317/FasL cells express functional FasL capable of inducing apoptosis in Fas-expressing cells.

In vitro interaction between BNL, CT26 and PA317/FasL or PA317/Contr cells

Using RT-PCR assay with specific primers derived from mouse Fas cDNA sequence we could demonstrate that both transformed liver cell line BNL cells and colon cancer CT26 cells did not express Fas. In addition, we observed that co-incubation of BNL or CT26 cells with either PA317/FasL or PA317/Contr did not affect the rate of ^{3}H-TdR incorporation into tumor cells, indicating that there is no direct interaction between Fas ligand on the surface of PA317/FasL and BNL or CT26 cells (data not shown).

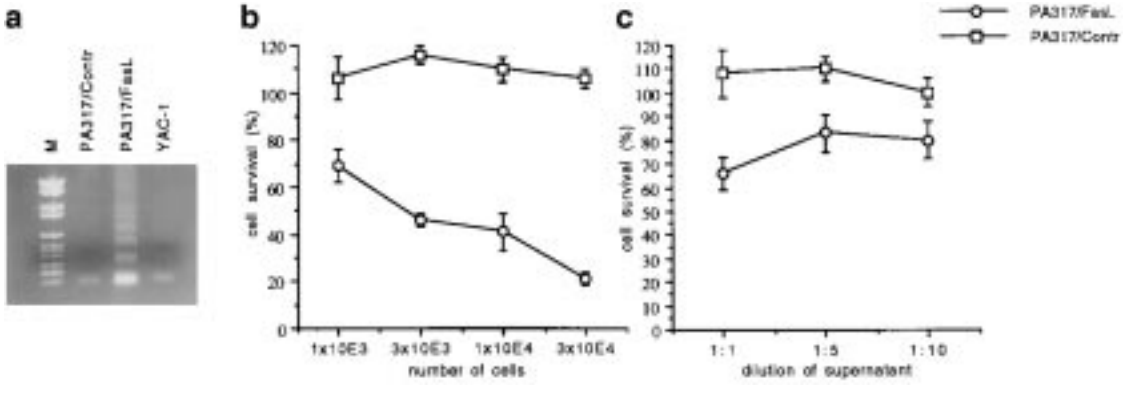

Figure 2 *Induction of apoptosis in Fas^{+} YAC-1 cells by incubation with PA317/FasL. (a) Apoptotic pattern of DNA fragmentation when YAC-1 cells were cocultured with PA317/FasL cells ; no fragmentation of DNA was observed when YAC-1 cells were incubated alone (YAC-1) or with PA317/Contr. M indicates molecular weight markers (b) JAM assay for detection of YAC-1 cell death when YAC-1 cells (5×10^{3}) were co-incubated with PA317/FasL or Pa317/Contr cells; figures in abscissa indicate the number of effector cells. (c) JAM assay for detection of YAC-1 cell death when they were incubated with supernatant from PA317/FasL or PA317/Contr cells. Each point represents mean ± s.d. of triplicate determinations.*

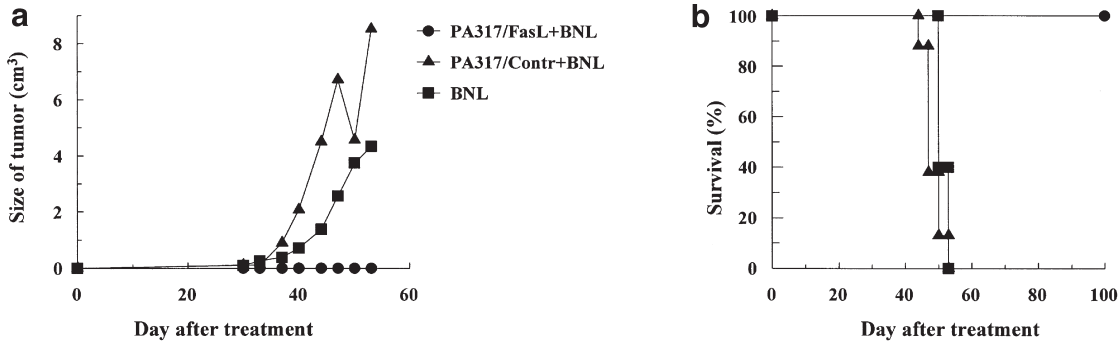

Figure 3 *Tumorigenicity of BNL tumor cells in vivo when they were mixed with PA317/FasL cells. BNL cells (5×10^6) alone, a mixture of BNL cells (5×10^6) and PA317/FasL cells (5×10^6) or PA317/Contr cells (5×10^6) were injected into Balb/c mice subcutaneously. (a) Tumor growth rate and (b) survival of animals receiving subcutaneous injection of BNL cells, a mixture of BNL+PA317/FasL cells or BNL+PA317/Contr cells at a ratio of 1:1. It can be observed that PA317/FasL cells abrogate the tumorigenicity of BNL cells, whereas PA317/Contr cells do not influence the rate of tumor growth.*

Effect of PA317/FasL cells on tumorigenicity of BNL and CT26 cells

As shown in Figure 3, PA317/FasL cells mixed at a ratio of 1:1 with BNL cells totally abrogated the tumorigenicity of neoplastic cells in such a way that no tumor appeared in all 12 inoculated animals. In contrast, in the group of animals given a mixture 1:1 of PA317/Contr and BNL tumors developed in all eight injected mice. The growing rate of tumors and animal survival were similar in mice receiving PA317/Contr+BNL or BNL alone. In animals given BNL cells mixed with PA317/FasL at a ratio of 2:1, 10:1 and 100:1 tumors appeared in one, four and five of five mice in each group, respectively (Table 1). In these animals the incidence of tumors as well as tumor growth rate increased as the proportion of PA317/FasL cells decreased (data not shown).

In a separate set of experiments we observed that, similarly to what was found with BNL cells, only one of eight animals developed a tumor when they received CT26 cells mixed with PA317/FasL at a 1:1 ratio. However, all eight animals developed tumors when they were given CT26 cells mixed with PA317/Contr cells at same ratio (Table 1).

In order to identify the effector cells involved in the eradication of the tumor cells, we performed histological examination of the inoculation site of BNL cells mixed with either PA317/FasL or PA317/Contr. In mice receiving a BNL and PA317/FasL cell mixture, a marked inflammatory response with predominance of infiltrating neutrophils and mononuclear cells was observed on day 1 (Figure 4a). Among inflammatory cells, cell debris was seen and BNL cells could not be clearly observed. In animals given BNL cells mixed with PA317/Contr cells, BNL cells were clearly identified surrounded by a margin of slight inflammation, with a few neutrophils and mononuclear cells (Figure 4d). On day 4, a massive infiltration of polymorphonuclear neutrophils and some mononuclear cells with a remarkable absence of BNL cells was observed at the site of injection of BNL cells with PA317/FasL (Figure 4b and c). In contrast, on day 4 obvious tumor growth with a rim of inflammatory reaction was noted in the group which received BNL cells together with PA317/Contr (Figure 4e). On day 7 no tumoral tissue at the site of tumor implantation was seen in animals injected with BNL cells mixed with PA317/FasL, whereas a solid nodule of tumoral cells was present in the group which received BNL plus PA317/Contr (Figure 4f).

Table 1 Effect of PA317/FasL on tumorigenicity of BNL and CT26 cells

PA317/FasL	*BNL*	*Tumor incidence*
5×10^6	5×10^6	0/12
2.5×10^6	5×10^6	1/5
5×10^5	5×10^6	4/5
5×10^4	5×10^6	5/5
PA317/Contr		
5×10^6	5×10^6	8/8
PA317/FasL	*CT26*	
5×10^5	5×10^5	1/8
PA317/Contr		
5×10^5	5×10^5	8/8

BNL and CT26 cells were mixed with various proportions of PA317/FasL or PA317/Contr cells. Cell mixture was injected s.c. into mice and tumor development was observed.

Induction of protective immunity against tumor cells

To study the protective antitumoral immunity induced by PA317/FasL, animals previously immunized with BNL and PA317/FasL or PA317/Contr cells at a ratio 1:1, were rechallenged on the opposite flank with 5×10^6 or 5×10^7 BNL cells or 5×10^5 CT26 cells on day 14 (short-term protection) or with 5×10^6 BNL cells on day 100 (long-term protection). None of the animals immunized with PA317/FasL+BNL and rechallenged with 5×10^6 and 5×10^7 BNL on day 14 ($n = 8$ per group) developed tumor growth. In contrast, rechallenge with CT26 cells of four mice immunized with BNL and PA317/FasL resulted in tumor formation in all cases. In animals treated with BNL and PA317/Contr cells, contralateral injection of BNL cells on day 14 led to tumor development in all cases ($n = 8$) (Table 2). In studies aimed at analyzing long-term protection, we found that 50% of mice immunized with BNL and PA317/FasL still exhibited protection against rechallenge with BNL cells 100 days after immunization (Table 2).

A specific antitumoral protective effect was also

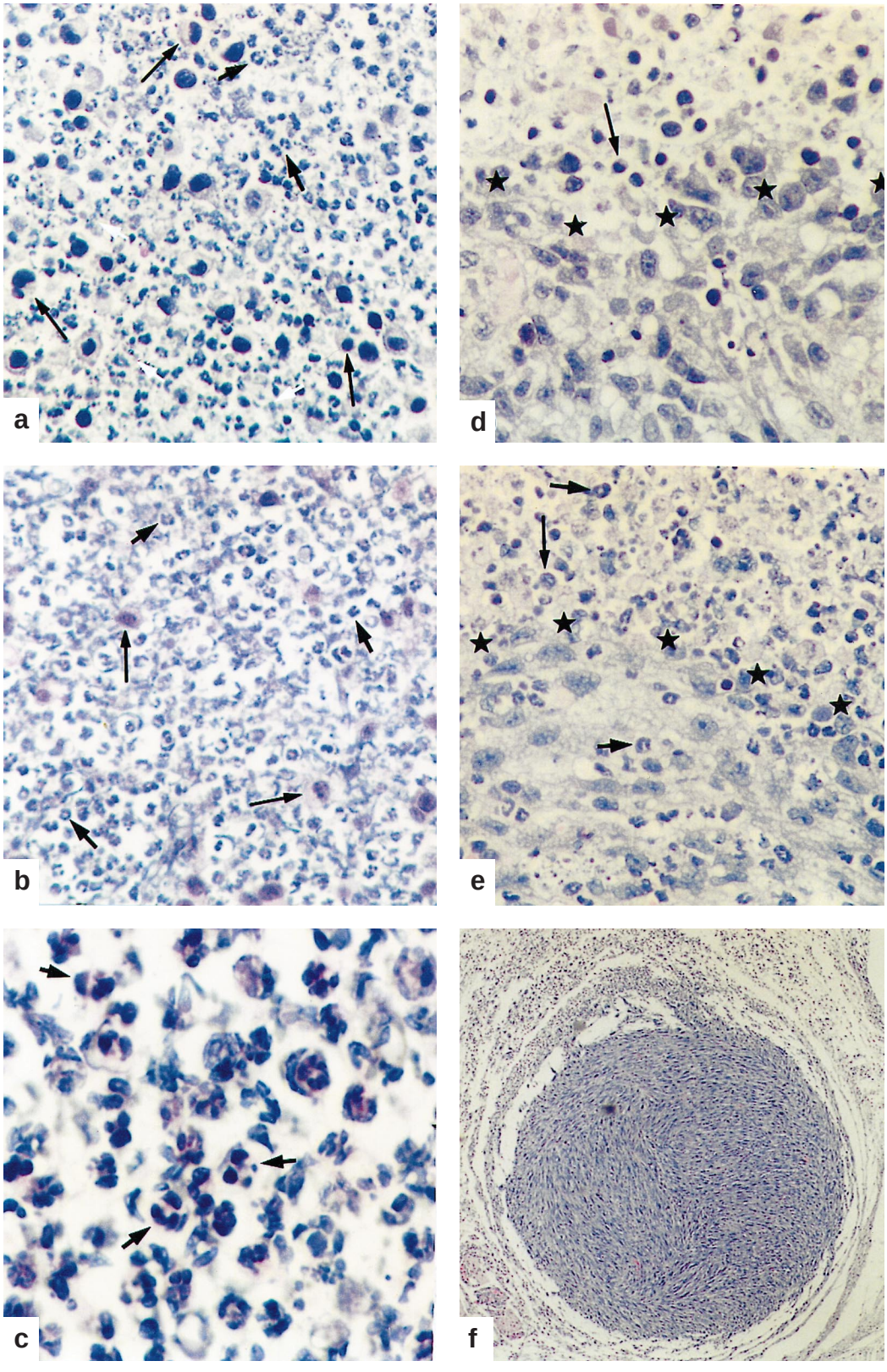

Figure 4 *Histological examination of the tumor implantation sites. Inflammatory response with predominance of polymorphonuclear neutrophils (thick short black arrows) and mononuclear cells (thin black arrows) in the infiltrate was observed in mice injected with BNL mixed with PA317/FasL cells on day 1 after inoculation. Among inflammatory cells cellular debris was seen (white arrows) (a). Massive infiltration of polymorphonuclear neutrophils and mononuclear cells was present at the site of injection of BNL cells mixed with PA317/FasL at day 4 (b). High magnification field showing polymorphonuclear neutrophils on day 4 in mice injected with BNL plus PA317/FasL (c). A cluster of BNL cells (below black stars) is surrounded by a rim of mild inflammatory reaction with a few neutrophils and mononuclear cells in animals inoculated with BNL cells mixed with PA317/Contr cells at day 1 after inoculation of the cell mixture (d). Formation of a small tumor nodule (below black stars) with mild peripheral inflammatory response was noted on day 4 in the group given BNL cells with PA317/Contr (e). A solid tumor nodule was observed on day 7 in the group which received BNL and PA317/Contr (f). Paraffin sections were stained with hematoxilin and eosin. Magnification a, b, d, e ×400, c ×1000, f ×100.*

Table 2 Tumor protective immunity induced by PA317/FasL

Priming	*Tumor incidence following rechallenge*		
	Day 14		*Day 100*
	BNL	*CT26*	*BNL*
BNL+PA317/FasL	5×10^6 0/8	5×10^5 4/4	5×10^6 2/4
	5×10^7 0/8	ND	ND
BNL+PA317/Contr	5×10^6 8/8	ND	ND
CT26+PA317/FasL	5×10^6 4/4	5×10^5 1/8	ND
CT26+PA317/Contr	ND	5×10^5 4/4	ND

ND, not done.

observed in mice immunized with CT26 and PA317/FasL cells, as a tumor appeared in only one of eight animals when they were rechallenged with CT26 cells on day 14 after immunization, while all four animals immunized with CT26 and PA317/FasL developed tumors when they were rechallenged with BNL cells (Table 2). All mice treated with CT26 plus PA317/Contr developed tumors after rechallenge with CT26 cells ($n = 4$).

Administration of monoclonal antibodies anti-CD4$^+$ and anti-CD8$^+$ resulted in a significant decrease of circulating CD4$^+$ and CD8$^+$ lymphocytes (93.4 and 85.2% reduction, respectively). Depletion of CD8$^+$ lymphocytes was associated with appearance of tumors in all rechallenged animals, whereas in animals given monoclonal antibodies anti-CD4$^+$ no tumor formation was observed (Table 3).

In vivo treatment of established tumor with PA317/FasL

In order to know whether antitumoral immunity induced by PA317/FasL could eliminate established BNL tumors, we treated established s.c. tumor (0.5 cm in diameter) by intratumor injection of PA317/FasL cells or by injection of a mixture of BNL and PA317/FasL cells at a distal site. Figure 5a shows that intratumor injection of 5×10^6 of PA317/FasL to previously established BNL tumors did not affect the growth of the tumors as compared with tumors injected with 5×10^6 PA317/Contr. In addition, we observed no differences in survival of tumor-bearing mice treated with intratumoral injection of either PA317/FasL or PA317/Contr (Figure 5b).

Injection of the mixture of PA317/FasL and BNL (5×10^6 and 5×10^6) cells to animals with previously established BNL tumors (0.5 cm in diameter) in the contralateral flank of the body resulted in significant inhibition of tumor growth, as compared with animals injected with a mixture of PA317/Contr and BNL cells (Figure 6a). The survival of mice with established tumors treated with a mixture of PA317/FasL and BNL cells was also longer ($P < 0.01$) than that of animals treated with a mixture of PA317/Contr and BNL cells (Figure 6b).

Table 3 Involvement of lymphocyte subtypes in tumor protective immunity induced by PA317/FasL

Priming	*Antibody treatment*	*Rechallenging*	*Tumor incidence*
BNL+ PA317/FasL	Anti-CD8	BNL	3/3
	Anti-CD4	BNL	0/3

Animals were primed at day −14 with a mixture of BNL+PA317/FasL and rechallenged with BNL cells at day 0. Anti-CD8$^+$ or anti-CD4$^+$ was given i.p. at days −3, −2, −1 and then every week for 50 days after challenge.

Discussion

Many discrepancies exist as to whether expression of FasL provides immune privilege or destruction of transplanted cells. The mechanisms that lead to one outcome or to the other are unknown. In some settings FasL expression leads to apoptosis of infiltrating lymphocytes resulting in immune tolerance. Thus, Sertoli cells, epithelial cells of the anterior chamber of the eye and some types of tumor cells, such as melanoma and colon cancer cells which express FasL, as well as myoblasts engineered to produce FasL can create an immunoprivileged environment.[13–18] Allogenic CT26 colon carcinoma cells expressing mouse FasL markedly reduced allogenic cytotoxic T lymphocytes and completely inhibited generation of alloantibodies of both IgM and IgG subclasses.[26] In contrast with these experimental findings, the presence of FasL on the surface of allogenic pancreatic islets failed to protect these islets from allogenic rejection and this reaction was found to involve the formation of a granulocytic infiltrate.[22] Introduction of human or mouse FasL cDNA into murine tumor cells did not affect tumor growth *in vitro* but caused rejection of the tumor by infiltrating neutrophils with subsequent induction of tumor-specific protective immunity.[21] More recent data indicate that adenovirus-mediated gene transfer of mouse FasL induced tumor regression in animal models implanted with Fas-positive and -negative tumor cells.[27]

In the light of the above data we decided to study whether allogenic fibroblasts engineered to express FasL are able to abolish tumorigenicity and induce tumor-specific protective immunity. We decided to transduce allogenic fibroblasts stably instead of syngenic cells because transduction with FasL of syngenic cells entails the risk of inducing a state of immunoprivilege.[16] Human FasL gene was chosen because of its ability to be functional in the soluble form, which could potentiate local inflammatory response, whereas mouse FasL loses its activity when it is cleaved from the membrane.[28] Furthermore, recombinant soluble human FasL is directly chemotactic for mouse neutrophils *in vitro*. From the practical point of view, allogenic fibroblasts with functional FasL seem to be more useful when thinking in terms of potential applications for treatment of tumors in humans, as allogenic fibroblasts can be easily prepared in large quantities. These cells can be mixed with irradiated tumor cells obtained from the patient and inoculated to induce a specific antitumoral immunity. When thinking in terms of practical applications, our strategy offers advantages in respect of the use of adenoviral vectors expressing Fas ligand, which can induce severe liver toxicity since hepatocytes express Fas constitutively[29] and also in respect of procedures based on direct transduction of tumor cells with Fas ligand since it is difficult to culture tumor cells from specific patients.

Our results show that fibroblasts engineered to express FasL and their supernatant were able to induce apoptosis

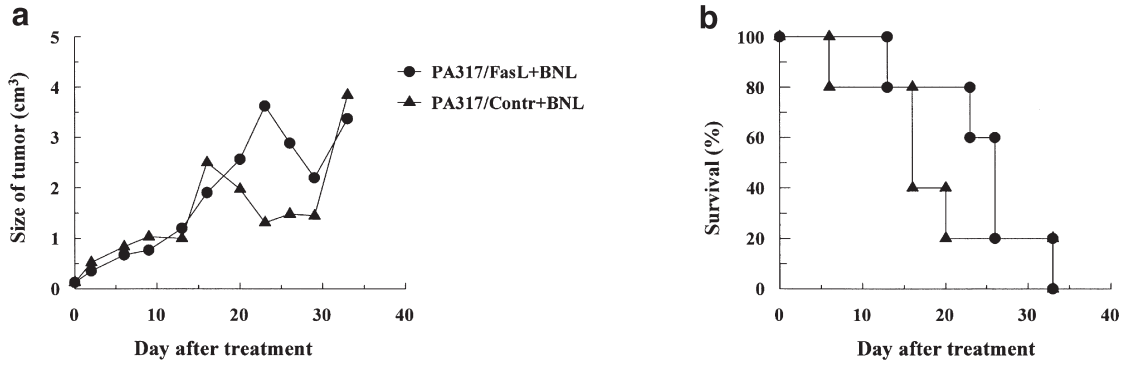

Figure 5 *Tumor growth rate and survival of animals with pre-established BNL tumors after intratumoral injection of PA317/FasL or PA317/Contr cells. Tumors were established by injection of 5×10^6 BNL cells subcutaneously. When tumors were 0.5 cm in diameter, they were treated by intratumor injection of either 5×10^6 PA317/FasL ($n = 5$) or PA317/Contr ($n = 5$) cells. After treatment both the size of the tumors and animal survival were documented. (a) Tumor growth rate and (b) survival of animals after treatment. No significant differences in tumor growth or survival rates were observed among mice which received intratumoral injection of PA317/FasL or PA317/Contr cells.*

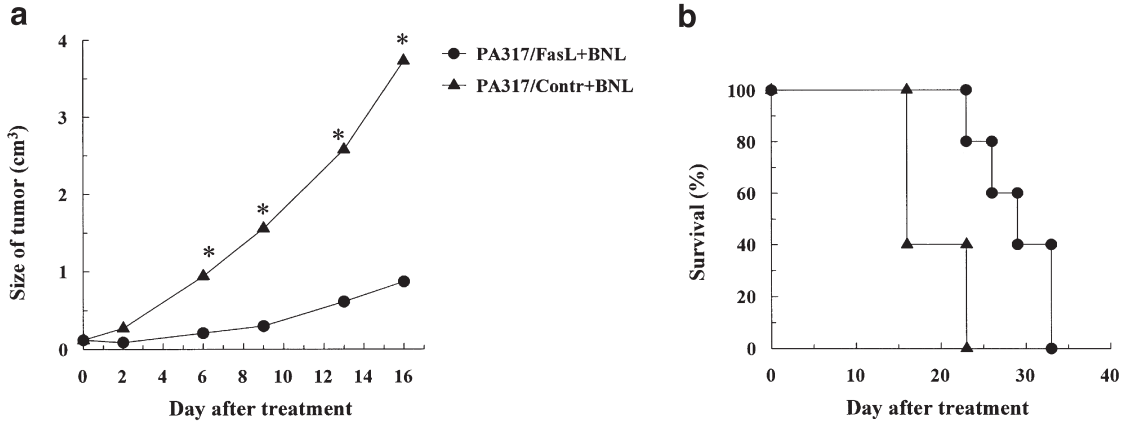

Figure 6 *Tumor growth rate and survival of animals with pre-established BNL tumors after contralateral injection of BNL+PA317/FasL or BNL+PA317/Contr. Tumors were established by injection of BNL cells at 5×10^6 s.c. When tumors were 0.5 cm in diameter, animals were treated by s.c. injection in the opposite flank with a mixture of 5×10^6 BNL cells with either 5×10^6 of PA317/FasL ($n = 5$) or PA317/Contr ($n = 5$) cells. (a) Tumor growth rate after treatment. Significant inhibition of tumor growth rate was noted in animals subjected to contralateral vaccination with BNL+PA317/FasL ($5 \times 10^6 + 5 \times 10^6$) as compared with BNL+PA317/Contr ($5 \times 10^6 + 5 \times 10^6$). *$P < 0.05$ (ANOVA test). (b) Survival of animals after treatment. Significant prolongation of survival was found in animals treated by contralateral vaccination with BNL+PA317/FasL as compared with BNL+PA317/Contr. $P < 0.01$ (log rank test).*

of Fas-bearing YAC-1 cells. Although PA317/FasL did not induce toxic effects on BNL or CT26 tumor cells *in vitro*, these tumor cells lose their tumorigenicity when they are implanted into syngenic mice together with PA317/FasL cells. This observation suggests that PA317/FasL cells trigger *in vivo* mechanisms responsible for tumor suppression. It has been shown that some cytokines such as tumor necrosis factor (TNF), even if they have no direct action on the target tumor cells *in vitro*, can induce antitumor reaction *in vivo* by potentiating nonspecific and/or specific host responses against tumors.[30,31] In our study, histologic examination of the site of implantation revealed the presence of a dense infiltration of neutrophils and mononuclear cells with disappearance of tumor cells in animals which received the mixture of tumor cells and PA317/FasL. In contrast, mice treated with BNL cells mixed with PA317/Contr exhibited progressive growth of neoplastic cells with formation of a tumor nodule surrounded by only a mild inflammatory reaction. This observation, which is similar to observations in previous reports using FasL-expressing tumor cells,[21] suggests that activation of neutrophils is a basic mechanism involved in tumor elimination in the system utilized in the present work.

Importantly, we observed that no tumor occurred after contralateral rechallenge with BNL cells in animals treated with BNL and PA317/FasL 14 days before. In contrast neoplastic growth developed in the same group of animals after rechallenge with a tumor cell line different from that used for immunization. These data point to a specific antitumoral immune response induced by treatment with a mixture of neoplastic cells and PA317/FasL cells. One hundred days after priming with a BNL and PA 317/FasL cell mixture 50% of animals still manifested protection. This effect appears to be due to the induction cytotoxic T cells since depletion of $CD8^+$ T cells abrogated the protection against tumor formation. Our unpublished data and reports in the literature[32] indicate that both BNL and CT26 are poorly immunogenic and express low levels of MHC class I antigens and no class II molecules. Thus, in accordance with previous studies,[21,27] our findings support the idea that immunity

induced by FasL against tumors seems not to depend on the immunogenicity of the malignant cells.

In our study no antineoplastic effect was observed by injecting PA317/FasL intratumorally. Different reasons might account for this lack of antitumoral effect: first, it may be possible that the viability of allogenic cells injected into the solid tumor nodule might not be good enough to cause biological effects; and second, the immunosuppressive microenvironment within an established tumor[33] might prevent neutrophil recruitment and the induction of an effective antitumoral immunity.

Interestingly, however, contralateral vaccination of animals with previously established BNL tumors with a mixture of BNL cells and PA317/FasL resulted in decreased tumor growth rate and in prolongation of animal survival, as compared with animals given BNL and PA317/Contr. These results indicate that treatment of animals with a mixture of BNL and PA317/FasL cells was able to induce immune responses which can not only eliminate tumor cells after rechallenge but also significantly inhibit the growth of pre-established tumors. This finding has important implications for the delineation of antitumoral strategies in humans aimed at preventing recurrence after surgical resection of neoplasms or at eliminating residual cancer cells after surgical removal of main, large tumors. These aims could be accomplished by vaccination of the patient after surgery using allogenic fibroblasts engineered to express FasL and irradiated tumor cells obtained at surgery from the same patient.

In summary, our data demonstrate that allogenic cells engineered with FasL are able to abrogate tumorigenicity of tumor cells *in vivo* by recruiting inflammatory cells. Allogenic fibroblasts expressing FasL are useful for antitumoral vaccination when administered in mixtures with syngenic tumor cells. This procedure protects against rechallenge with neoplastic cells and inhibits growth of established tumors by eliciting a specific antitumor immune response. These data suggest that local expression of FasL can be used as a tool for immunotherapy of cancer.

Materials and methods

Animals, cell culture and reagents

Six- to eight-week-old Balb/c female mice were used in the study. They were purchased from Charles River (Barcelona, Spain). During the experimental period animals were housed under standard conditions. Methylcholanthrene epoxide-transformed mouse liver cell line BNL 1ME A.7R.1 (BNL) from Balb/c mouse, YAC-1 mouse lymphoma cell line and PA317 mouse fibroblast cells from Swiss mouse were obtained from American Type Cell Collection (ATCC, Rockville, MD, USA). Colon cancer (CT26) cell line from Balb/c mouse was a kind gift from Dr K Brand, Max-Planck-Institut fur Biochemie (Germany). The cell lines were cultured at 37°C, 5% CO_2 in DMEM (BNL, PA317) or RPMI 1640 (CT26, YAC-1) medium supplemented with 10% fetal calf serum, 2 mm l-glutamine and penicillin/streptomycin. All media and supplements were from Biological Industries (Kibbutz Beit Haemek, Israel) and BioWhittaker (Vervier, Belgium).

Plasmid construction

The human Fas ligand (FasL) cDNA was released from pBX-hFL1 plasmid[1] (a kind gift from S Nagata) by *Xba*I cutting and was cloned into the *XbaI* site of pCl/neo eukaryotic expression vector plasmid (obtained from Promega, Madison, MI, USA). The obtained plasmid included FasL gene under control of the CMV promoter together with the linked dominant biochemical selection marker gene neomycin, a geneticin-resistance gene driven by simian virus 40 (SV40) promoter, allowing the selection of transfected cells in geneticin-containing medium. The orientation of FasL insert relative to promoter was confirmed by enzyme digestion.

Stable transfection

A newly created plasmid (pCl/FasL) or an empty plasmid (pCl/neo) were transfected into PA317 fibroblast cell line using calcium phosphate transfection method. Briefly, the 1×10^5 PA317 cells were seeded per well of six-well plate. On the next day, cells were transfected with 10 μg of plasmid DNA for 16 h. Two days after transfection, cells were cultured in geneticin (G418)-containing medium (GibcoBRL, Barcelona, Spain) at a concentration of 800 μg/ml for 2 weeks. Approximately 10–15 colonies from each transfection were picked up for screening of FasL expression. One colony from pCl/FasL transfection that expressed high levels of FasL and another from pCl/neo transfection that did not express FasL were chosen for further studies and were designated as PA317/FasL and PA317/Contr.

RNA isolation and RT-PCR

To identify clones with stable expression of FasL, total RNA was isolated from all colonies using commercial kit Ultraspec RNA (Biotex, Houston, TX, USA). For RT-PCR, cDNA was synthesized from 1 μg of total RNA in 10 μl of volume using M-MuLV reverse transcriptase. PCR amplification was performed with 4.8 μl cDNA, 100 pmol each of oligonucleotide primer, 200 mm dNTP, 3 mm $MgCl_2$, and 2.5 units *Taq* polymerase in PCR buffer (Biotaq; Ecogen, Barcelona, Spain) in a total volume of 50 μl for 30 cycles. Each cycle included denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and primer extension at 72°C for 1 min. The sense and antisense PCR primers for human FasL gene used were CAGCTCTTCCACCTACAGAAGG (5′ to 3′, nt 371–392 corresponding to published sequence from GenBank accession no. U11821) and CAAAATTGACCAGAGAGAGCTC (5′ to 3′, nt 872–851). 502 bp of FasL fragment was obtained after PCR amplification. As a control we used amplification of the β-actin gene.[34] To analyze Fas expression in BNL and CT26 cells we used 1 μg of total RNA for reverse transcription, followed by amplification using mouse Fas primers in above conditions for 30 cycles. The sense and antisense PCR primers for mouse Fas gene used were GCTGGCTCACAGTTAAGAGT (5′ to 3′, nt 86–105 corresponding to the published sequence)[35] and CAACCATAGGCGATTTCTGG (5′ to 3′, nt 565–546). 480 bp of Fas fragment was obtained after PCR amplification.

To measure the growing rate of PA317/FasL and PA317/Contr, cells were seeded in triplicate in a 96-well dish at 1×10^3, 5×10^3, 1×10^4 cells per well for 48 h and then were labeled with ^{3}H-TdR (methyl-^{3}H thymidine; Amersham Iberica, Madrid, Spain) for 8 h. Finally, cells

were harvested and measurement as well as calculation was given as above.

Assessment of DNA fragmentation in target cells

The function of Fas ligand in stably transfected PA317 cells was assessed by quantification of DNA fragmentation of target cells by the JAM test.[36] The adherent PA317/Fasl and PA317/Contr cells were seeded in triplicate into a flat-bottomed 96-well microtiter plate at 1×10^3, 3×10^3, 1×10^4, 3×10^4 cells and were incubated in complete DMEM medium at 37°C for 24 h. Target Fas-positive YAC-1 cells were labeled by prior incubation with 10 μCi of ^{3}H-TdR in complete RPMI 1640 medium at 37°C for 16 h. Labeled target cells were washed and 5×10^3 of cells were added to the seeded effector cells in a final volume of 100 μl of complete RPMI 1640 medium. After coculture at 37°C for 8 h, the cells were removed from the wells by pipetting up and down five times and were collected by filtration on to glass fiber filters using a 96-well filtration unit. The cells were hypotonically lysed, and fragmented DNA was washed through the filter four times with 0.25 ml water. The radioactivity of intact chromosomal DNA retained on each filter was measured by liquid scintillation counting. Specific cell killing was calculated using the following equation:

$$\%\ \text{specific killing} = (S - E/S)\times 100$$

where E (experimental) is c.p.m. of retained DNA in the presence of effector cells and S (spontaneous) is c.p.m. of retained DNA in the absence of effector cells. Clones of PA317/FasL with the most pronounced activity and one of PA317/Contr were selected for further studies. To study the activity of the soluble form of Fas ligand, the supernatants from 3-day confluent cultures of PA317/Fasl and PA317/Contr were harvested and filtered through 0.45-μm pore filters and stored at 4°C until the experiment. Preparation of target YAC-1 cells was the same as above. On the day of the study, three dilutions of supernatant were prepared (1:1, 1:5 and 1:10) and 5×10^3 target YAC-1 cells were added to a final volume of 100 μl in complete RPMI 1640 medium on a 96-well dish. Then, cells were incubated for 8 h and harvested, c.p.m. measurement and calculation were made as stated above.

For analysis of DNA fragmentation by electrophoresis, 1×10^6 YAC-1 cells were cocultured in complete RPMI 1640 medium with a confluent monolayer of previously washed PA317/Fasl or PA317/Contr in 10-cm plates for 8 h. Then, floating YAC-1 cells were harvested, washed once and resuspended in lysing buffer (10 mm Tris/HCl pH 8.0; 10 mm EDTA; 0.5% Triton X). Tubes were then transferred to ice and the cells were lysed for 5 min. After centrifugation at 13 000 *g*, the supernatant was treated with 100 μg/ml RNAse (Boehringer Mannheim, Penzberg, Germany) for 2 h at 37°C. Then, SDS and proteinase K (Sigma, St Louis, MO, USA) were added to a final concentration of 0.5% and 200 μg/ml, respectively. The extracts were left for 2 h at 50°C, and subsequently supernatants were carefully collected. The low molecular weight DNA was extracted twice in phenol and phenol/chloroform, and ethanol precipitated. Finally, the DNA was resuspended in TE buffer and loaded on to 1.5% agarose gel.

In vitro interaction between BNL, CT26 and PA317/FasL or PA317/Contr cells

BNL or CT26 cells were labeled with ^{3}H-TdR and then were trypsinized, washed three times, and mixed with either PA317/FasL or PA317/Contr. The mixed cells (10^4 of tumor cells and 10^4 PA317/FasL or PA317/Contr) were seeded into 96-well plates in triplicate and incubated for 8 h. Finally, cells were harvested and measurement of radioactivity and calculations were performed as stated above.

In vivo evaluation of tumor growth

BNL cells as well as CT26 cells were mixed at a ratio of 1:1 with either PA317/FasL ($n=12$ for BNL and $n=8$ for CT26) or PA317/Contr ($n=8$ for both BNL and CT26), and the mixture inoculated s.c. into one flank of Balb/c mouse. The number of injected cells was 5×10^6 for BNL and 5×10^5 for CT26 cells. In additional experiments 5×10^6 BNL cells were mixed with PA317/FasL or PA317/Contr cells at the ratios 1:1, 1:2, 1:10 and 1:100 ($n=5$, in all cases).

To study protective immunity, Balb/c mice were inoculated with BNL cells or with CT26 cells mixed at a ratio of 1:1 with either PA317/FasL or PA317/Contr in one flank of the body, and 14 days later animals were rechallenged with 5×10^6 ($n=8$) or 5×10^7 ($n=8$) BNL, or 5×10^5 ($n=4$) CT26 in the opposite flank of the body. Four additional mice were rechallenged with 5×10^6 BNL cells 100 days after receiving the mixture of BNL cells with PA317/FasL at a ratio of 1:1.

For treatment of established tumors, mice received 5×10^6 BNL cells. After 30–40 days animals developed tumors of 0.5 cm in diameter. In some experiments we injected intratumorally 5×10^6 of PA317/FasL ($n=5$) or PA317/Contr ($n=5$) cells and in other experiments animals were injected s.c. in the opposite flank of the body with 5×10^6 BNL cells mixed at a ratio of 1:1 with PA317/FasL ($n=5$) or PA317/Contr ($n=5$) cells. Tumor growth was assessed by periodically measuring perpendicular diameters with a caliper. Tumor volume was calculated from the square of the longest diameter (a) multiplied by a smaller diameter (b) of the tumor ($V=a\times b^2$). Mice free of tumors 100 days after challenge were classified as survivors. Animals with tumors that exceeded 1.5 cm in two perpendicular diameters or 2.0 cm in one were killed for humanitarian reasons.

In vivo depletion of lymphocytes and tumor incidence

L3T4 ($CD4^+$)-specific rat anti-mouse hybridoma GK1–5 and ($CD8^+$)-specific rat anti-mouse hybridoma H35.17.2 were used to obtain anti-$CD4^+$ and anti-$CD8^+$ antibodies. Ascitic fluid was obtained from nude mice pristane-primed and injected with 10^6 hybridoma cells. Antibodies were prepared by precipitation with ammonium sulphate and dialyzed against phosphate-buffered saline. The protein concentration was assessed by measurement of the OD at 280 nm. Mice, three in each group, were depleted of $CD4^+$ or $CD8^+$ cells by i.p. injection of 300 μg of anti-$CD4^+$ or anti-$CD8^+$ antibodies,[37] on days –3, –2, –1 and every 7 days for 50 days, following rechallenge with 5×10^6 BNL cells (this was done on day 0). On day –14 all animals were primed with the mixture of BNL cells and PA317/FasL at a ratio of 1:1 in the flank contralateral to that of the site of rechallenge. The efficiency of the depletion was assessed by flow cytometry on day 10.

Tumor growth incidence was assessed twice a week for 50 days.

Histological examination

Mice were inoculated subcutaneously with 5×10^6 BNL cells mixed with PA317/FasL or PA317/Contr cells at a ratio of 1:1. On days 1, 4 and 7, the inoculated site was dissected, fixed in 10% neutral-buffered formalin, and embedded in paraffin. Sections of 5 μm were stained with hematoxylin and eosin.

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