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Tumor formation by a murine macrophage cell line immortalized in vitro by v-*raf* and v-*myc* oncogenes

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Summary. Murine bone marrow cells immortalized in vitro by the J2 recombinant retrovirus bearing the v-*raf* and v-*myc* oncogenes have the functional and phenotypic characteristics of macrophages. The present study was designed to determine whether these cells are tumorigenic in athymic or euthymic mice. One cloned cell line (GG2EE), that had been previously derived and characterized was used for this purpose. The results demonstrated that GG2EE cells were tumorigenic in allogeneic athymic BALB/c mice at doses of 1×10^4 to 1×10^7 cells per mouse regardless of the route administration. All mice utlimately died of progressive tumor growth. Conversely, the GG2EE cells were nontumorigenic or transiently tumorigenic in syngeneic euthymic C3H/HeJ mice. Further studies in BALB/c athymic mice demonstrated that the GG2EE cells were directly tumorigenic since ascites tumors (GG2EE-V) that developed expressed the H-2^k surface phenotype of the injected GG2EE cells, excluding the possibility that the J2 virus constitutively produced by GG2EE cells caused in vivo transformation and therefore tumors of host cell origin. The in vivo passaged cells continued to express the M1/69, MAC-1, MAC-2, F4/80, Fc receptor and Ly5.1 antigens characterically expressed on the parental line. Biological properties including interferon-γ-induced Ia expression, phagocytosis, and activation for cytotoxicity were also retained following in vivo passage. These results demonstrated that J2 virus-immortalized GG2EE cells were directly tumorigenic in athymic mice in vivo and that the macrophage phenotype was maintained in these neoplastic cells. These observations suggest that this tumor model may be valuable for the study of macrophage function as well as therapeutic approaches to oncogen-expressing retrovirus-induced tumors.

Introduction

The possible relationship between oncogene expression and neoplastic transformation is currently an area of intensive investigation. For logistical reasons many studies have been performed using murine models employing retroviruses to carry viral oncogenes (v-*raf* and v-*myc*) into cells. These studies have demonstrated the ability of v-*raf* and v-*myc* to cooperate in cellular immortalization/transformation in vitro as well as in vivo [2, 9]. We have previously reported that the introduction of two oncogenes (v-*raf* and v-*myc*) into bone marrow cells resulted in cell immortalization [3, 4]. Neither oncogene alone was able to induce cell growth, thus suggesting that in this experimental model a cooperation between v-*raf* and v-*myc* was needed to allow immortalization. Increasing evidence exists to indicate that cell transformation resulting in in vivo neoplastic growth is a complex process consisting of several discrete steps [1, 9, 10, 14, 15]. Thus, our studies were designed to determine whether the presence of the v-*raf* and v-*myc* oncogenes in the immortalized cells (GG2EE cells) was sufficient to render these cells tumorigenic in vivo. The data presented in this paper demonstrate that the v-*raf* and v-*myc*-immortalized GG2EE cells are tumorigenic in athymic mice, but not in syngeneic euthymic hosts, and that the macrophage phenotype is maintained following in vivo growth.

Materials and methods

Mice – C3H/HeJ and Balb/nucle mice were obtained from the Animal Production Area of the National Cancer Institute – Frederick Cancer Research Facility. Mice were between 7 and 10 weeks old when used.

Cell lines – The GG2EE cell line was established previously by infecting in vitro freshly isolated bone marrow cells with the J2 recombinant retrovirus carrying the v-*raf* and v-*myc* oncogenes [3]. Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, glutamine (2 m*M*), penicillin (100 μ/ml), and streptomycin (100 μg/ml). The P815 cell line (a DBA/2 mastocytoma) was maintained in vitro in RPMI 1640 supplemented with glutamine (4 m*M*), gentamicin (50 μg/ml), and serum (10%). All reagents were obtained from Gibco, Grand Island, NY. The GG2EE cells were routinely monitored, and found to be positive for the H-2 antigen which is the haplotype of the C3H/HeJ mouse strain from which they derive. Moreover, GG2EE cells retained the lipopolysaccharide unresponsiveness characteristic of macrophages from C3H/HeJ mice [5], confirming that they did not drift from the original genetic makeup in a detectable way.

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Reagents. The reagents used in the present studies were recombinant murine interferon-γ (IFNγ) (Genentech, South San Francisco, Calif.; heat-killed *Listeria monocytogenes* (HKLM), kindly provided by Dr. R. D. Schreiber (Washington University, St. Louis, Mo.), and zymosan A (Sigma, St. Louis, Mo.). The following antibodies were used for flow cytometry analysis: M1/69, 2.4G2, and Ly 5.1, kindly provided by Dr. B. Mathieson (Biological Response Modifiers Program, Frederick, Md.), MAC-1 and MAC-2 antibodies were purchased from Boehringer Mannheim Biochemicals, Indianapolis, Ind.; F4/80 was generously supplied by Dr. S. Gordon; Thy 1.2 antibodies were purchased from Cederlane, Hornby, Ontario, Canada; anti-H-2^k and H-2^D antibodies were provided by New England Nuclear, Boston, Mass.; and anti-IA antibodies (14.4.4) were provided by Dr. B. Tartakovsky. Fluorescein isothiocyanate conjugated (FITC)-anti-immunoglobulins (goat anti-rat and goat anti-mouse immunoglobulins) were purchased from Cappel Laboratories (Malvern, Pa.). The FITC-protein A was obtained from Pharmacia, Inc. (Piscataway, NJ).

Tumorigenicity in mice. Tumorigenicity of the GG2EE cell line was determined by injection of 10^4 to 10^7 cells/mouse. Cells had been previously washed twice and resuspended in phosphate-buffered saline. Injections were done i.p., i.v. or intrafoot pad (IFP). Mice were monitored for tumor appearance and survival for 8–12 weeks following injection.

Analysis of surface markers. Analysis of surface markers on the cells was performed by flow cytometry. Briefly, in vitro-immortalized cell lines or in vivo-collected ascitic cells were washed twice in phosphate-buffered saline containing 0.5% bovine serum albumin and 0.1% sodium azide and resuspended to 2×10^7 cells/ml. All staining procedures were carried out at 4° C. Each cell suspension (50 µl) was mixed with an equal volume of antibodies and incubated for 45 min. The cells were then washed three times, and 10 µl of the appropriate antibody added. After another 45 min, the cells were washed three times and analyzed on a cytofluorograph system 30-H with a 2150 computer (Ortho Diagnostic, Westwood, Mass.) capable of simultaneously measuring forward angle light scatter (488 nm) and red (>600 nm) and green (530 nm) fluorescence. Cells were illuminated by a 4 W argon laser (Lexel, Palo Alto, Calif.) emitting 500 MW of light. Cells analyzed for immunofluorescence were selected by forward and right angle light scatter. Dead cells (<1%) were identified by the uptake of propidium iodine (red fluorescence) and excluded from green fluorescence analysis. A green fluorescence histogram of 1,000 channel resolution was collected for 10,000 viable cells for each sample analyzed. The percentage of positive cells was calculated by histogram subtraction. The medium fluorescence intensity of specifically stained cells was also determined to provide a measure of the average fluorescence of the cell populations.

Phagocytosis. Zymosan A particles were resuspended in mouse serum (5 mg/ml) and then added to the cell cultures at a final concentration of 100 µg/ml. Phagocytosis was allowed to take place overnight at 37° C. Thereafter, the excess zymosan was removed by centrifugation of the cell suspension on a Ficoll cushion at 1100 rpm for 10 min. The cells at the interface were recovered and washed. The zymosan uptake was directly evaluated in Giemsa-stained cytospin preparations.

Cytotokixity assays. The GG2EE cell-mediated cytotoxicity was assessed in a 24 h ^{111}In release assay as detailed elsewhere [17]. Briefly, GG2EE cells (10^5 cells/well) were plated in 96-well plates (Dynatech, Alexandria, Va.), and IFNγ (100 units/ml) and/or HKLM ($\approx 2 \times 10^7$ particles/ml) were added as stimulating agents at a final volume of 200 µl/well. After overnight incubation, the cells were washed by spinning the plates at 1100 rpm for 10 min, the supernatant removed and 200 µl/well of warm complete medium was added. This washing procedure was repeated three times. Following an overnight stimulation, GG2EE cells were then tested against ^{111}In-labeled P815 target cells in a 24-h cytotoxicity assay, at various effector to target ratios.

The results of the cytotoxicity assays were expressed as percentage cytotoxicity, calculated from the average of triplicate samples according to the following formula:

$$\frac{\text{cpm with effector cells} - \text{cpm spontaneous release}}{\text{total cpm incorporated in target cells} - \text{cpm spontaneous release}}$$

The spontaneous release from target cells alone was between 5% and 15% of the total radioactivity incorporated.

Statistical analysis. Statistical analysis of the data was done using Student's *t*-test.

Results

Tumorigenic potential of GG2EE cells in BALB/c athymic and C3H/HeJ syngeneic euthymic mice

Various doses of GG2EE cells ranging from 1×10^4 to 1×10^7 cells were injected i.p. or s.c. into BALB/c athymic mice. The mice were then monitored for tumor appearance and survival. All mice developed tumors at the site of injection and died irrespective of the route of inoculation (Table 1). The mean survival time was inversely related to the number of GG2EE cells initially injected with even 10^4 cells/mouse being uniformly lethal. Routinely, s.c. injection of GG2EE cells resulted in a 2- to 3-fold increase in mean survival time as compared to i.p. injection of equivalent amounts of cells, although all mice eventually succumbed to tumor growth. These data indicated that GG2EE cells were highly tumorigenic when injected into BALB/c athymic mice either i.p. or s.c.

Because athymic mice are immunoincompetent with regard to T cell-mediated specific immunity, experiments were performed to determine whether GG2EE cells could be tumorigenic in T lymphocyte competent syngeneic C3H/HeJ mice. The results shown in Table 2 demonstrated that C3H/HeJ were resistant to challenge with 1×10^6 GG2EE cells, a dose at least 100-fold greater than that required for tumor formation in athymic mice (Table 1). Although tumors initially developed at some sites of injection (IFP or s.c.) all tumors regressed within 17 to 24 days. This failure to grow progressively occurred irrespective of the route of tumor cell injection. Overall these results suggested that evasion of T cell-mediated immune responses was required for GG2EE cell growth in vivo.

Table 1. Tumorigenic potential of GG2EE cells in BALB/c athymic mice

Inoculum size[a]	MST (days)[b]	
	i.p.	s.c.
10^7	13	33
10^6	17	31
10^5	20	49
10^4	23	53

[a] All groups contained 7 mice and all mice in the experiment died as a result of progressive tumor growth
[b] MST = mean survival time

Stability of surface phenotype and functional ability of GG2EE cells following tumor growth in vivo

We have previously shown that GG2EE cells produce J2/leuk virus [3, 4]. Such a virus transforms 3T3 fibroblasts in vitro [7] and induces tumors in vivo in newborn mice [12]. Therefore, experiments were performed to investigate whether tumors which developed locally in GG2EE cell-injected mice were actually due to growth of the transplanted GG2EE cells or to host cells transformed by J2/leuk virus infection. To distinguish between these possibilities GG2EE cells, which express the H-2^k haplotype, were injected into BALB/c athymic (H-2^D) mice and the expression of either H-2 haplotype on the cells collected from the peritoneal cavity 12 days after injection was tested. Table 3 shows the mean fluorescence values obtained by FACS analysis of individual ascites (GG2EE-V) cells from 6 mice. Greater than 95% of the cell population recovered from in vivo passage expressed the H-2^k marker, whereas only 4% expressed H-2^D antigens. Furthermore, we found that 100% of the clones (14 tested so far) established in vitro from ascities cultures were H-2^K positive (data not shown). These data indicated that the tumors which developed locally in GG2EE cell-injected mice were derived directly from in vivo growth of the in vitro-immortalized cells.

The in vivo passaged ascites cells also retained the same pattern of surface markers expressed by the parental GG2EE cells. MAC-1, MAC-2, and Ly 5.1 were expressed on most cells in both populations, as indicated by the percentage number of positive cells and by the mean fluorescence values (Table 3). M1/69, F4/80, and Fc receptor were also present in comparable amounts in GG2EE and GG2EE-V cells.

Table 2. Effect of GG2EE cell injection on the survival of syngeneic C3H/HeJ and allogeneic BALB/c athymic mice

Route of injection[a]	MST (days)[b]	
	C3H/HeJ	BALB/c athymic
i.v.	>90 (1/7)[a]	19 (7/7)
i.p.	>90 (0/7)	17 (7/7)
s.c.	>90 (0/7)	39 (7/7)
intrafoot pad	>90 (0/7)	44 (7/7)

[a] Mice were injected with 10^6 cells at day 0
[b] Values in parentheses indicate the number of dead mice/total number of treated mice

Table 3. Pattern of surface markers of GG2EE cells and in vivo-passaged GG2EE-V ascites cells

Surface markers	% Positive cells[a, b]	
	GG2EE	GG2EE-V
M1/69	72 (283)	68 (371)
MAC-1	91 (325)	84 (511)
MAC-2	81 (273)	78 (480)
F4/80	56 (231)	61 (382)
Fc receptor	58 (285)	49 (329)
Thy 1.2	1 (241)	2 (329)
Ly 5.1	99 (784)	97 (844)
H-2^D	Not done	4 (304)
H-2^K	Not done	95 (602)

[a] % positive cells was evaluated by flow cytometry, as detailed in *Materials and methods*
[b] The values represent the mean % positive cells obtained by testing individually ascites cells from 6 mice. The numbers in parentheses indicate the mean fluorescence values of the positive cells

In addition to the surface phenotype stability, we also examined functional stability of the in vivo-passaged GG2EE-V cells as compared to the in vitro-cultured GG2EE cells. Previous studies have shown that GG2EE cells have a macrophage-like phenotype, respond to IFNγ with augmented adherence, enhanced phagocytic ability, and expression of surface Ia antigens, and become cytotoxic upon treatment with IFNγ plus HKLM [5]. We found that the in vivo-growing ascites cells were indistinguishable from the in vitro-immortalized parental cell line in their functional activities. As illustrated in Table 4, GG2EE-V cells were noncytotoxic, exhibited limited phagocytic ability against zymosan particles (44% of the cells were phagocytic based on a phagocytosis index of 5), and had low Ia antigen expression (9% positive). However, upon 48 h treatment with IFNγ, their phagocytic ability

Table 4. Functional activities of GG2EE-V ascites cells

Functional activities	Stimuli			
	None	IFNγ	HKLM	IFNγ and HKLM
Phagocytosis[a]				
% Phagocytic cells	44	38	N.T.	N.T.
Phagocytosis index	5	22	N.T.	N.T.
Ia antigen expression[a]				
% Positive cells	9	92	N.T.	N.T.
mean fluorescence	374	875	N.T.	N.T.
Cytotoxic activity[b]				
E:T = 4:1	2.4	3.7	1.9	29.8
E:T = 20:1	2.0	2.5	2.7	41.4
E:T = 100:1	3.1	2.4	2.5	58.7

[a] Phagocytosis and Ia antigen expression were evaluated in GG2EE-V cells cultured in medium or in the present of interferon-γ (IFNγ) (100 units/ml) for 48 h as detailed in *Materials and methods*
[b] Cytotoxic activity of GG2EE-V cells was measured after overnight exposure to IFNγ (100 units/ml) and/or heat-killed *Listeria monocytogenes* (HKLM) ($\approx 2 \times 10^7$ particles/ml) as detailed in *Materials an methods*
E:T effector:target ratio

was strongly enhanced, with 98% of the cells being phagocytic and the phagocytosis index increasing to 22. Treatment of GG2EE-V cells for 48 h with IFNγ also resulted in augmented adherence (data not shown) and in the induction of surface Ia antigen (>90% positive cells). Furthermore, when tested for cytotoxic activity against ^{111}In-labeled P815 target cells, the GG2EE-V cells, like the parental in vitro-maintained GG2EE cells [5], acquired cytotoxic activity following exposure to IFNγ plus HKLM, and the levels of cytotoxicity obtained were directly related to the effector:target ratios used (Table 4).

Overall, our results indicated that the in vitro-immortalized GG2EE cell line was able to generate tumors in athymic mice. The in vivo passage did not affect the phenotypical and functional properties of the GG2EE cells, suggesting that the v-*raf*/v-*myc*-driven tumor cell remained stable regardless of the various immunoregulatory signals occurring in vivo.

Discussion

In the experiments reported here, we have shown that the in vitro-immortalized GG2EE cell line, is tumorigenic in mice. The GG2EE macrophage-like cell line has been generated in vitro by introduction of v-*raf* and v-*myc* oncogenes into freshly isolated murine bone marrow cells by injection with the recombinant J2 retrovirus. Normal bone marrow cells die quickly when cultured in vitro in the absence of exogenous growth factors. The J2 virus immortalized monocytic bone marrow cells by inducing proliferation and promoting continuous growth of these cells. Although the GG2EE cells produce the J2 retrovirus [3] which induces lymphomas, erythroblastomas, and fibrosarcomas in newborn mice [12], the proliferation of the GG2EE cells themselves was responsible for formation of the tumors rather than the growth of J2 virus-transformed host tissue. This was suggested by the fact that the J2 virus failed to induce tumors in adult mice (unpublished observation). However, we eliminated the possibility that the tumors arose in nude mice as a result of J2 virus transformation of host cells by demonstrating that the tumor cells collected from the peritoneal cavity of GG2EE cell-injected BALB/c athymic mice (H-2^d) bore the donor H-2 haplotype (H-2^k).

We have previously classified GG2EE cells as macrophages, based on their morphological and phenotypical properties [3–5]. Moreover, despite the high levels of v-*raf* and v-*myc* mRNA and their ability to grow continuously, we found that GG2EE cells retain many functional activities [4, 5], similar to those expressed by terminally differentiated peritoneal macrophages [18]. We think that the J2 virus had provided a proliferative stimulus to a bone marrow macrophage precursor still able to differentiate to a functionally mature stage but retain proliferative ability in vitro. The need for two oncogenes to induce primary cell growth in vitro is in agreement with the findings of Land et al. [10] and Ruley [13]. They have described the in vitro immortalization of primary rat embryo fibroblasts and baby rat kidney cells by two cooperating oncogenes, such as the EJ-*ras* and the v-*myc* oncogenes, or the EJ-*ras* and the adenovirus Ela gene, but not by a single oncogene alone. In those experimental models, the in vitro immortalization was accompanied by in vivo tumorigenicity. Tumorigenic ability was also acquired by preneoplastic hamster fibroblasts or by a CSF-1-dependent macrophage cell line, following introduction of EJ-*ras* [8] or v-*fms* [18] oncogenes, respectively. Overall these results demonstrate the multistage nature of oncogene-induced cell immortalization/transformation. The fact that GG2EE cells grow in mice indicates that the effects of J2 virus go beyond cell immortalization and result in transformation of bone marrow macrophages or their progenitors. This finding is consistent with a previous observation that the J2 virus transformed 3T3 cells such that they could induce tumors in mice [7]. However, our studies provide the first evidence that introduction of v-*raf* and v-*myc* into freshly isolated bone marrow cells causes transformation which renders the cells tumorigenic.

Our studies have shown that GG2EE cells were highly tumorigenic in athymic nude mice, but they grew poorly or not at all in syngeneic immunocompetent mice. These results suggest that GG2EE cells are highly immunogenic, probably because of viral antigens expressed on their surface [3]. Thus it is possible that tumor growth can be inhibited by a variety of T cell-dependent mechanisms, including anti-J2 antibodies, specific cytotoxic T lymphocytes, or growth inhibitory cytokines such as IFNγ. Alternatively, T lymphocytes might be stimulated to elaborate interleukin 2 or IFNγ which might induce natural killer-mediated antitumor responses.

GG2EE cells respond to IFNγ in vitro with augmented phagocytosis, enhanced adherence, and expression of Ia antigens. Moreover, they acquire cytotoxic activity upon treatment with IFNγ plus HKLM [5]. The in vivo passage of GG2EE cells consistently results in the maintenance of their morphological and inducible functional properties. Interestingly, the fact that GG2EE-V cells retain low baseline phagocytic ability and undetectable Ia surface antigen expression, suggests that the levels of IFNγ produced in vivo at the site of tumor growth are below the threshold needed to modulate the surface phenotype and function of GG2EE cells. This raises the possibility that since GG2EE cells are very sensitive to IFNγ which also happens to be a growth inhibitory signal for leukocytes in vitro [8], the lack of tumor growth observed in syngeneic immunocompetent mice may be partially due to direct IFNγ-mediated growth inhibitory effects on GG2EE cells. Experiments are in progress to determine the relative contributions of cytokines and host cells in the inhibition of GG2EE growth in syngeneic mice.

Overall, we believe that the tumorigenic potential exhibited by GG2EE cells, their susceptibility to IFNγ, and their differential tumorigenic abilities in normal or immunoincompetent mice, make this experimental model suitable to study the events involved in the establishment and/or regression of viral oncogene-induced tumors. In addition, since some combinations of biological response modifiers inhibit transcription of viral mRNA in vitro [6], this model may prove useful in studying the effects of these biological response modifier combinations on viral mRNA production in macrophages and virus production by macrophages in vivo.

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