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Complement-Dependent Control of Teratoma Formation by Embryonic Stem Cells

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The fetus has pluripotent stem cells that when transferred to mature individuals can generate tumors. However, for reasons yet unknown, tumors form rarely in the fetus and/or the mother during normal gestation. We questioned whether the complement system might protect against tumor formation by pluripotent stem cells. Murine embryonic stem cells were notably more susceptible than cardiomyocytes differentiated from those cells to lysis by complement in heterologous and homologous sera. Treatment of embryonic stem cells with heterologous serum averted tumor formation after residual cells were transplanted into mice. Confirming the importance of homologous complement in preventing formation of tumors, untreated embryonic stem cells formed tumors more quickly in C3-deficient than in wild-type mice. Susceptibility of embryonic stem cells to complement required an intact alternative pathway and was owed at least in part to a relative deficiency of sialic acid on cell surfaces compared with differentiated cells. Susceptibility to complement and resistance to tumors was inversely related to the number of cells transferred. These findings show that formation of tumors from embryonic stem cells is controlled in part by the alternative pathway of complement and suggest that susceptibility to complement might represent a general property of pluripotent stem cells that can be exploited to prevent tumor formation.

Materials and Methods

Sources and culture of embryonic stem cells

The 129Scv embryonic stem cells were a gift from Dr. R. Bram (Mayo Clinic College of Medicine, Rochester, MN). C57BL/6 and BALB/c × 129 embryonic stem cells were obtained from Open Biosystems. Murine embryonic stem cells were cultured feeder-free on 0.1% gelatin-coated flasks in DMEM supplemented with 15% embryonic stem cell-qualified FCS, 15.0 mM HEPES, 50 mg/L penicillin G, 50 mg/L streptomycin, 2.0 mM glutamine (Invitrogen Life Technologies), 100 μg/ml nonessential amino acids, 0.110 g/L MEM sodium pyruvate (Invitrogen Life Technologies), 100 μg/ml normocin (Invivo-Gen), and 1000 U/ml leukemia inhibitory factor (ESGiRO; Chemicon International), an embryonic stem cell medium. The medium was exchanged daily, and cultures were passaged every 2 days. Embryonic stem cells were considered undifferentiated based on the expression of OCT-4, detected by semiquantitative RT-PCR, alkaline phosphatase by enzymatic assay (Sigma-Aldrich), and stage-specific embryonic Ag-1 by FACS. Embryonic stem cells were periodically tested for the ability to form teratomas in immunodeficient Rag2Δcγc−/−γc−/− mice (Taconic Farms).

Differentiation of embryonic stem cells

Differentiation of embryonic stem cells was induced by formation of embryoid bodies using the hanging drop culture method previously described (6). Embryonic stem cells were cultured in hanging drops containing 300 cells per 30 μl of embryonic stem cell medium without leukemia inhibitory factor and allowed to form embryoid bodies for 5 days. After 5 days, embryoid bodies were collected and cultured in 96-well flat-bottom plates coated with 0.1% gelatin for an additional 7 days. After 12 days of culture,

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areas of spontaneously beating cardiomyocytes were observed in the cultures. In some experiments, cardiomyocytes were enriched by Percoll (Roche) gradient centrifugation as previously described (7).

Assay of complement-mediated cellular lysis

The susceptibility of cells to lysis by complement was measured as follows. Cells to be tested were labeled overnight with 1 μCi/well of [14C]sodium chromate (Amersham Biosciences). After labeling and washing, the cells were exposed to 25% heat-inactivated human serum (one donor), as a source of Abs, followed by serial dilutions of human serum, as a source of complement, or freshly obtained murine and neonatal diluted in DMEM for 4 h at 37°C and 5% CO2. In some experiments, cells were treated with 1.0 U/ml phosphatidylinositol-specific phospholipase C (PIPLC)3 (Sigma-Aldrich) for 1 h before addition of complement. The supernatant from cells treated with human serum was collected and the radioactivity measured (cpm test sample) using a Wallac scintillation counter (PerkinElmer). Cells remaining in wells following collection of the supernatant were lysed with 1% Triton X-100 and residual radioactivity measured (cpm residual cell sample). The percentage of specific lysis was calculated as (cpm test sample)/(cpm test sample + cpm residual cell sample)) – (cpm spontaneous release)/(cpm spontaneous release + cpm spontaneous release residual))/((cpm Triton X-100 – cpm residual cells Triton X-100) – (cpm spontaneous release)/(cpm spontaneous release + cpm spontaneous release residual)) × 100. In some experiments, cells were treated with 10 U/ml neuraminidase (Sigma-Aldrich) in buffer containing 50 mM sodium acetate, 0.9% NaCl and 0.1% CaCl2 (pH 5.5), for 1 h before treatment with complement.

Expression of Crry

The expression of Crry/p65 was measured as follows. Undifferentiated embryonic stem cells or purified cardiomyocytes derived from embryonic stem cells were detached from culture dishes with 0.5% trypsin/EDTA. The cells were incubated with biotin-conjugated rat anti-mouse Crry/p65 Abs or isotype control (rat IgG2a, IgG1) Abs diluted 1/5000 in 0.5% BSA/PBS. The immune complexes were measured by ELISA, as previously described (8, 9). Cells were exposed to 25% heat-inactivated human serum followed by serial dilutions of normal human serum for 1 h at 37°C. Treated cells were fixed with 0.1% glutaraldehyde for 5 min at 4°C and incubated for 1 h with 1% BSA in PBS to block nonspecific protein binding. Complement components were detected using mouse mAbs specific for human C1q, C3b, C5b. Factor B (Quidel), a neoantigen of the membrane attack complex (a gift from A. F. Michael, University of Minnesota, Minneapolis, MN) or a monoclonal FITC-conjugated goat Ab specific for murine C3 (ICN/Cappel) diluted 1/1000 in 0.5% BSA/PBS. Bound Abs were measured by incubating the cells with alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma-Aldrich) Abs diluted 1/5000 in 0.5% BSA/PBS. The immunochromatic reactions were detected by adding p-nitrophenylphosphate in 0.1 M diethanolamine (Sigma-Aldrich) and measuring absorbance (405 nm) using Power Wave Microplate Reader (Bio-Tek Instruments). Fluorescence of goat anti-murine C3 Abs was measured (excitation/emission = 490/530 nm) using a FL600 Microplate Fluorescence Reader (Bio-Tek Instruments).

Analysis of the expression of sialic acid and heparan sulfate

Embryonic stem cells maintained in an undifferentiated state or induced to differentiate for 12 days were grown on Permanox two-well chamber slides (Nalge Nunc International). The cells were washed three times with PBS and allowed to dry in air at room temperature. Biotinylated Maackia amurensis lectin II (1/25) or FITC-conjugated Sambucus nigra lectin (1/40; Vector Laboratories) were diluted in 5% BSA/PBS and applied to slides for 30 min at room temperature. Slides were washed three times in PBS, fixed for 10 min in Karnovsky’s solution, and washed three times in PBS. FITC-Ultravidin (Leinco Technologies) diluted 1/200 in 5% BSA/PBS was applied to M. amurensis lectin II-treated slides for 30 min, washed three times, and coverslipped with a 1/8 dilution of Vectashield-DAPI (4′,6-diamidino-2-phenylindole, 1.5 μg/ml; Vector Laboratories) in PBS (pH 8.6). Heparan sulfate was detected with FITC-conjugated mouse monoclonal anti-human heparan sulfate Abs (US Biological) diluted 1/25 in 0.5% BSA/PBS. Slides were examined using an epifluorescence microscope. Digital images were obtained using a high-resolution CCD digital camera (SPOT II; Diagnostic Instruments) and accompanying software.

Transplanting embryonic stem cells in mice

Embryonic stem cells were transplanted into C57BL/6, Rag2−/−,γc−/−(Taconic Farms), and C57BL/6 (The Jackson Laboratory) mice on a C57BL/6 background as follows. Embryonic stem cells suspended in PBS were injected s.c. between the scapulae of mice. Mice were examined every day for the presence of tumors and followed for 3 mo or until tumor growth necessitated the sacrifice of the animal for humane reasons. The largest length and width of tumors was determined using a micrometer and multiplied to calculate surface area.

Statistical analysis

Results are expressed as the mean ± SEM. An unpaired two-tailed Student’s t test was used to compare means with a value p < 0.05 were considered significant. Nominal data were compared using the Chi square or Fisher’s exact test where appropriate.

Results

Sensitivity of embryonic stem cells and cells differentiated from embryonic stem cells to complement-mediated injury

To determine whether complement might control the growth of embryonic stem cells and formation of tumors, we asked to what extent embryonic stem cells are sensitive to complement-mediated cytotoxicity and whether this sensitivity changes during differentiation. To address these questions, embryonic stem cells from 129SvJ mice were incubated with human serum as a source of anti-mouse Abs and complement (10, 11). The sensitivity of the embryonic stem cells to complement was compared with the sensitivity of cardiomyocytes and cells from embryoid bodies differentiated from embryonic stem cells. As shown in Fig. 1A, embryonic stem cells were highly susceptible to complement-mediated lysis compared with cardiomyocytes that had been grown from those cells or even cells derived from embryoid bodies. For example, when exposed to 25% human serum, 73.8 ± 5.0% of embryonic stem cells underwent lysis, whereas under the same conditions only 18.8 ± 4.3% of cardiomyocytes differentiated from embryonic stem cells and 7.3 ± 2.5% of differentiated cells from embryoid bodies underwent lysis. Neither undifferentiated nor differentiated embryonic stem cells were killed by 25% heat-inactivated human serum alone (data not shown). Embryonic stem cells from C57BL/6 and BALB/c × 129 strains of mice were similarly sensitive to killing by complement (data not shown).

We next asked whether murine embryonic stem cells activate homologous complement. To address this question, C57BL/6 embryonic stem cells were treated with C57BL/6 serum and the deposition of C3 and lysis of the cells were measured. Murine C57BL/6 embryonic stem cells treated with normal homologous serum had substantially more C3 bound to the cell surface than murine fibroblasts (Fig. 1B). This reaction was specific for C3, as it was not observed when cells were incubated with serum from C3−/− mice. Deposition of complement in diluted homologous serum could lyse the cells as 16.1 ± 3.1% of embryonic stem cells treated in this way were lysed, whereas only 4.9 ± 2.1% murine fibroblasts were lysed (Fig. 1C) (p < 0.05). Lysis was owed to complement as serum from C3−/− mice did not lyse murine embryonic stem cells.

Complement as a barrier to tumor formation

Having found that undifferentiated embryonic stem cells are highly susceptible to complement-mediated lysis, we asked whether complement might pose a barrier to formation of teratomas after transplantation of embryonic stem cells. To address this question, we treated 2.5 × 106 129SvJ embryonic stem cells with 15% human

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3 Abbreviation used in this paper: PIPLC, phosphatidylinositol-specific phospholipase C.
pressed as mean or C57BL/6 fibroblasts were labeled with 1.0 Ci/ml [51Cr]sodium chromate and incubated sequentially with 25% heat-inactivated human serum, as a source of anti-mouse Abs, and then with serial dilutions of normal human serum, as a source of complement. Specific lysis was determined as described in Materials and Methods. Experiments were performed in triplicate and results are expressed as mean ± SEM. Similar results were obtained when cells were not pretreated with heat-inactivated human serum. B, Activation of homologous complement by murine embryonic stem cells. Murine C57BL/6 embryonic stem cells or murine C57BL/6 fibroblasts were treated with 50% normal murine serum for 4 h. Specific lysis was determined as described in Materials and Methods. Results are expressed as mean ± SEM of three independent experiments. C, Sensitivity of murine embryonic stem cells to homologous complement-mediated lysis. The target cells were labeled with 1.0 μCi/ml [51Cr]sodium chromate and incubated sequentially with 25% heat-inactivated human serum, as a source of anti-mouse Abs, and then with serial dilutions of normal human serum, as a source of complement. Specific lysis was determined as described in Materials and Methods. Results are expressed as mean ± SEM of three independent experiments. **, p < 0.001.

_FIGURE 1_ Sensitivity of embryonic stem cells to complement-mediated lysis. A, Sensitivity of murine embryonic stem cells to heterologous complement-mediated lysis. Murine 129SvJ embryonic stem cells, 129SvJ embryonic stem cells induced to form embryoid bodies and differentiated for 12 days, and cardiomyocytes purified from embryonic stem cells that had differentiated for 12 days were tested for susceptibility to complement-mediated lysis. The target cells were labeled with 1.0 μCi/ml [51Cr]sodium chromate and incubated sequentially with 25% heat-inactivated human serum, as a source of anti-mouse Abs, and then with serial dilutions of normal human serum, as a source of complement. Specific lysis was determined as described in Materials and Methods. Experiments were performed in triplicate and results are expressed as mean ± SEM. Similar results were obtained when cells were not pretreated with heat-inactivated human serum. B, Activation of homologous complement by murine embryonic stem cells. Murine C57BL/6 embryonic stem cells or murine C57BL/6 fibroblasts were treated with 50% normal murine serum or 50% C3−/− murine serum for 30 min. The treated cells were fixed, and the deposition of C3 was measured by fluorescent immunomassay. Results are expressed as mean ± SD and are expressed as a representative of three independent experiments. C, Sensitivity of murine embryonic stem cells to lysis by homologous complement. Murine C57BL/6 embryonic stem cells or C57BL/6 fibroblasts were labeled with 1.0 μCi/ml [51Cr]sodium chromate and incubated with 50% normal murine serum for 4 h. Specific lysis was determined as described in Materials and Methods. Results are expressed as mean ± SEM of three independent experiments. **, p < 0.001.

serum (a condition in which ~70% of cells are killed and ~30% survive) or heat-inactivated human serum and then transferred the remaining cells into Rag2−/−γc−/− mice, which lack T cells, B cells, and NK cells but have an intact complement system (12), and the formation of teratomas was monitored (Fig. 2A). One hundred percent of mice receiving transplants of undifferentiated embryonic stem cells not treated with complement or undifferentiated embryonic stem cells treated with heat-inactivated human serum developed teratomas, whereas only 20% of mice receiving cells treated with human serum developed teratomas (p < 0.05). These results suggest that complement can control formation of teratomas from embryonic stem cells and suggests a potential strategy for limiting that complication in transplants of cells differentiated from embryonic stem cells.
that question, we transplanted $1 \times 10^6$ C57BL/6 embryonic stem cells into C3$^{-/-}$ or wild-type mice and monitored the formation of teratomas. As Fig. 2B shows, embryonic stem cells formed teratomas more rapidly in C3$^{-/-}$ mice than in wild-type mice.

Although complement clearly slowed the formation of tumors following administration of embryonic stem cells, it did not prevent the eventual formation of tumors under the conditions we had used. One potential explanation for this failure was that, in contrast to the dispersal of pluripotent stem cells in the fetus and mother, cells injected as a bolus formed aggregates in which some cells would be protected from direct contact with complement. To test this possibility (among others) we asked whether the formation of teratomas in wild-type mice depended on the number of embryonic stem cells transferred. As Fig. 2C shows, $1 \times 10^6$ embryonic stem cells always generated teratomas C3$^{-/-}$ and in wild-type mice, whereas $1 \times 10^5$ embryonic stem cells generated teratomas only in C3$^{-/-}$ mice and not in wild-type mice. These results confirm that complement poses a barrier to tumor formation by embryonic stem cells but that this barrier has a threshold that can be overcome when larger numbers of cells are transferred. Because the transfer of pluripotent stem cells is unlikely to occur as a “bolus” under natural conditions, we suspect the transfer of the smaller number of cells better represents the conditions in which stem cells reach a gravid female during pregnancy.

Mechanism of complement activation on embryonic stem cells

To specifically test the use of the classical, lectin, and alternative pathways in the activation of human complement in this system, embryonic stem cells were exposed to anti-mouse Abs and then to C2-depleted or Factor B-depleted human serum and cellular lysis was measured. 129SvJ embryonic stem cells treated with C2-depleted serum were lysed to the same extent as embryonic stem cells treated with normal human serum ($p > 0.05$), suggesting the classical and lectin pathways are not needed for activation of complement on embryonic stem cells. In contrast, lysis of Ab-primed 129SvJ embryonic stem cells was significantly decreased when the source of complement lacked Factor B ($p < 0.01$) (Fig. 3A), suggesting that the alternative pathway was needed for complement to be activated. Unlike 129SvJ embryonic stem cells, C57BL/6 embryonic stem cells did exhibit some sensitivity to the classical pathway or lectin pathways as lysis by C2-depleted human serum was less than by fully constituted serum ($p < 0.05$); however, C57BL/6 embryonic stem cells exhibited a profound decrease in cellular lysis when Factor B-depleted serum was used as a source of complement ($p < 0.0001$) (Fig. 3B). These results suggest that embryonic stem cells are highly susceptible to injury by the alternative pathway of complement.

To determine which pathway of complement prevented formation of tumors by undifferentiated embryonic stem cells, 2.5 $\times 10^6$ 129SvJ embryonic stem cells treated with 15% human serum depleted of either C2 or Factor B were transplanted into Rag2$^{-/-}$ mice, and the formation of tumors was monitored. Teratomas formed in 80% of mice that received embryonic stem cells treated with Factor B-depleted human complement and in only 30% of mice that received embryonic stem cells treated with C2-depleted serum (Fig. 2A). These results suggest that the alternative pathway of human complement can prevent pluripotent stem cells from forming teratomas.

Although the control of teratomas in immunodeficient mice clearly requires C3 and the alternative pathway of complement, it is possible that in immunocompetent mice, cytotoxicity might be amplified by the production of Abs that activate the classical or alternative pathways of complement. To exclude this possibility, we tested whether B cells contribute to the barrier to teratoma formation. Toward that end, $1 \times 10^6$ C57BL/6 embryonic stem cells were transplanted into JH$^{-/-}$ mice, which lack B cells (13), and the rate of tumor formation was compared with the rate observed when the same number of stem cells were transplanted into wild-type mice. As Fig. 2D shows, teratomas formed at the same rate in JH$^{-/-}$ mice and wild-type mice. These results suggest that functions of B cells and Abs do not protect against teratoma formation from pluripotent stem cells.

Complement regulation and the susceptibility of embryonic stem cells to complement

The susceptibility of embryonic stem cells to complement could reflect control of the complement cascade or intrinsic susceptibility to lysis or both. As a first approach to this question, we measured the amounts of various components of human complement that fix to the surface of undifferentiated murine embryonic stem cells and cardiomyocytes differentiated from those cells. As Fig. 4 shows, after exposure to human serum for 1 h, undifferentiated embryonic stem cells had more iC3b, Factor B, C5b, and membrane attack complex deposited on the surface than cardiomyocytes differentiated from those cells, but approximately the same amount of C1q. Similar results were obtained when cells were treated for 30 min or longer. Although the control of teratomas in immunodeficient mice clearly requires C3 and the alternative pathway of complement, it is possible that in immunocompetent mice, cytotoxicity might be amplified by the production of Abs that activate the classical or alternative pathways of complement. To exclude this possibility, we tested whether B cells contribute to the barrier to teratoma formation. Toward that end, $1 \times 10^6$ C57BL/6 embryonic stem cells were transplanted into JH$^{-/-}$ mice, which lack B cells (13), and the rate of tumor formation was compared with the rate observed when the same number of stem cells were transplanted into wild-type mice. As Fig. 2D shows, teratomas formed at the same rate in JH$^{-/-}$ mice and wild-type mice. These results suggest that functions of B cells and Abs do not protect against teratoma formation from pluripotent stem cells.

**FIGURE 3.** Mechanism of complement activation on embryonic stem cells. 129SvJ (A) and C57BL/6 (B) embryonic stem cells were labeled with 1.0 $\mu$Ci/ml $^{51}$Cr sodium chromate and incubated with 25% heat-inactivated human serum as a source of anti-murine Abs followed by 12.5% normal human complement (C), human serum depleted of C2 (−C2), human serum depleted of Factor B (−Factor B), or human serum heated to 56°C for 30 min to inactivate complement (AC) as a source of complement. Specific lysis was determined as described in Materials and Methods. The results show that lysis of embryonic stem cells requires the alternative pathway of complement. ****, $p < 0.01$.

**FIGURE 4.** Complement activation on embryonic stem cells and cardiomyocytes differentiated from embryonic stem cells. 129SvJ embryonic stem cells and cardiomyocytes differentiated from those cells were incubated with 10% heat-inactivated human serum as a source of anti-mouse Abs and 2.5% normal human serum as a source of complement. The deposition of C1q, iC3b, Factor B, C5b, and the membrane attack complex (MAC) was measured by ELISA. Incubations were performed in triplicate and the results are expressed as mean ± SD. The results show that iC3b, Factor B, C5b, and membrane attack complex but not C1q are deposited to a greater extent on embryonic stem cells than on cardiomyocytes differentiated from embryonic stem cells.
2 h (data not shown). The higher levels of complement deposited on embryonic stem cells suggest that higher susceptibility of these cells to complement may be caused at least in part by decreased regulation of the complement cascade.

We next asked whether heightened susceptibility of embryonic stem cells to complement might be caused simply by the absence or lower levels of expression of Crry, the main complement regulatory protein, or CD59, which regulates the membrane attack complex in mice. To address the first possibility, we compared the expression of Crry on undifferentiated embryonic stem cells with that of cardiomyocytes differentiated from the embryonic stem cells. Undifferentiated embryonic stem cells and cardiomyocytes differentiated from embryonic stem cells expressed similar levels of Crry measured by flow cytometry (Fig. 5A). Hence, differential expression of Crry would not explain the relative sensitivity of undifferentiated embryonic stem cells to complement. To exclude a role for CD59, we treated the various cells with PIPLC, which cleaves the lipid anchor of CD55 and CD59, releasing the proteins from cells, and then measured susceptibility of the treated cells to complement (Fig. 5B). Treatment of undifferentiated embryonic stem cells with PIPLC did not significantly increase susceptibility to complement-mediated lysis. Treatment of cardiomyocytes differentiated from embryonic stem cells with PIPLC did significantly (p < 0.05) increase the susceptibility of the cells to complement-mediated lysis, as expected; however, the level of

susceptibility was still far less than that of undifferentiated embryonic stem cells. These results suggest that the dramatically heightened susceptibility of embryonic stem cells to complement is not owed to the absence or differential expression of Crry or CD59.

**Properties of embryonic stem cells that increase susceptibility to complement**

Because differential expression of complement regulatory proteins did not explain the heightened susceptibility of embryonic stem cells to the alternative complement pathway, we questioned whether another cell surface protein might do so. Because CR2 can activate the alternative pathway of complement (14, 15), we asked whether it might be expressed on undifferentiated embryonic stem cells. Undifferentiated embryonic stem cells did not express mRNA for CR2, as measured by semiquantitative RT-PCR (data not shown). Thus, expression of CR2 could not explain the increased activation of the alternative complement pathway by undifferentiated embryonic stem cells.

Sialic acid and heparan sulfate heighten the function of factor H and thus decrease alternative complement pathway activation on cell surfaces (16, 17). Hence, we tested whether decreased expression of these saccharides could explain heightened susceptibility of embryonic stem cells to lysis. Undifferentiated embryonic stem cells exhibited strikingly lower levels of cellular binding of M. amurensis lectin II and S. nigra lectin, lectins that specifically bind sialic acid, than cardiomyocytes differentiated from embryonic stem cells (Fig. 6). In contrast, both undifferentiated embryonic stem cells and cardiomyocytes differentiated from embryonic stem cells bound anti-heparan sulfate Abs to the same extent (Fig. 6). These results suggest that heightened susceptibility of undifferentiated embryonic stem cells to complement might be owed in part to lower levels of sialic acid on cell surfaces. To determine to what extent expression of sialic acid might explain differential susceptibility to complement, we treated differentiated cells with neuraminidase, which specifically removes sialic acid from oligosaccharides and then tested susceptibility to complement. As shown in

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**FIGURE 5.** The potential role of complement regulatory proteins in the greater resistance of differentiated cells than embryonic stem cells to complement-mediated injury. Whether differential expression of complement regulatory proteins accounts for the decreased susceptibility of differentiated cells to complement-mediated cytotoxicity compared with embryonic stem cells was tested by measuring expression of Crry/p65 and determining whether the susceptibility of the cells to complement-mediated lysis was increased by removal of phosphatidylinositol-linked complement regulatory proteins. *A*, Expression of Crry/p65 on embryonic stem cells and differentiated cells. Embryonic stem cells (129SvJ) and cardiomyocytes differentiated from the embryonic stem cells were incubated with biotin-conjugated anti-Crry/p65 Abs or isotype control for 30 min on ice and then with PE-conjugated streptavidin. The binding of the Abs was measured by FACS. *B*, Sensitivity to lysis of cells treated with PIPLC. Embryonic stem cells (129SvJ), cardiomyocytes differentiated from embryonic stem cells and porcine endothelial cells, used as a positive control, were treated with 1.0 U/ml PIPLC for 1 h to release CD59 and CD59 from cell surfaces. After treatment with PIPLC, cells were exposed to 10% heat-inactivated human serum, as a source of anti-mouse Abs, and 6.25% normal human serum, as a source of complement, for 4 h. Specific lysis was measured as described in Materials and Methods. Results are expressed as mean ± SEM of three independent experiments. The results show that differences in the expression of complement regulatory proteins cannot explain the susceptibility of embryonic stem cells compared with differentiated cells.

**FIGURE 6.** Expression of sialic acid and heparan sulfate on embryonic stem cells and cardiomyocytes differentiated from embryonic stem cells. The ability of cells to activate the alternative pathway of complement is controlled in part by the negative charge of cell surfaces; accordingly expression of sialic acid and heparan sulfate, the main sources of negative cell surface charge, were tested. The expression of sialic acid by embryonic stem cells and cardiomyocytes differentiated from embryonic stem cells for 12 days was measured by staining the cells with biotinylated *Maackia amurensis* lectin II (MALII) lectins, which specifically recognize sialic acid, followed by FITC-conjugated ultravidin or FITC-conjugated *Sambucus nigra* (SNA) lectins, which also specifically recognize sialic acid. The expression of heparan sulfate was measured by staining cells with FITC-conjugated mouse mAbs specific for heparan sulfate. Magnification, ×200. These results show that embryonic stem cells express low levels of sialic acid and that the expression of sialic acid increases during differentiation. Neither embryonic stem cells nor cardiomyocytes differentiated from embryonic stem cells expressed significant amounts of heparan sulfate.
Fig. 7, treatment of cardiomyocytes with neuraminidase significantly increased susceptibility to complement-mediated lysis. This result suggests that the susceptibility of embryonic stem cells to complement is at least partially owed to a relative deficiency of sialic acid on the surface of these cells.

Discussion
Teratomas occur infrequently in the newborn (18–20), and teratomas of fetal origin, other than choriocarcinoma, are rare in gravid or parous females (21, 22). However, embryonic stem cells engrafted in mature, histocompatible or immunodeficient individuals usually generate teratomas (23). In this study, we show that the formation of teratomas depends on the number of cells transferred and that when limiting numbers of embryonic stem cells are seeded in a mouse, the complement system, particularly the alternative pathway of complement, controls formation of teratomas. These findings suggest that susceptibility to complement may be an intrinsic property of embryonic stem cells and this property may protect the fetus and potentially the mother from formation of tumors.

Pluripotent stem cells exist in the fetus throughout development, enter the maternal circulation at various times, and contribute to tissues in adult mammals (24). Ariga et al. (25) found that by 36 wk gestation, each of 25 healthy pregnant women had fetal cellular DNA, as measured by PCR for the Y chromosome, in their circulation. Srivatsa et al. (26) found male cells by fluorescent in situ hybridization for the Y chromosome in the thyroid follicles of the mothers of male offspring. Khosrotehrani et al. (27) used a similar method to detect male cells in the cervix, intestine, thyroid, and gallbladder of previously gravid mothers. Because the male cells were detected in many mature tissues, they concluded that these cells derived from pluripotent “pregnancy-associated progenitor cells.” The identity of the pregnancy-associated progenitor cell remains unknown; however, some believe these cells resemble embryonic stem cells (28). If pluripotent stem cells can be found in the fetus during much of fetal life and enter the mother, one might reasonably ask what prevents the formation of teratomas from these cells, and why does this mechanism fail to protect the recipient of transplanted embryonic stem cells.

One mechanism why pluripotent stem cells do not form tumors in the mother (or fetus) is that the stem cells might be short lived. Khosrotehrani et al. (29) bred male C57BL/6 mice transgenic for GFP with nontransgenic DBA/2 female mice and studied the mothers carrying GFP+ fetuses during gestation and after parturition. During gestation, gravid female mice always had fetal GFP+ cells; however, following parturition, GFP+ fetal cells were completely cleared from tissues and the circulation within 1 wk. Bonney and Matzinger (30) also found that fetal cells detected by quantitative PCR are rapidly cleared from the circulation of mice and that clearance is mediated by the maternal immune system. However, the mechanism(s) by which the fetal cells were cleared was not determined.

One mechanism that might explain the clearance of fetal stem cells from the mother is cell-mediated immunity. We recently found that embryonic stem cells of a given batch form teratomas in syngeneic mice but not in fully allogeneic mice (C. A. Koch and J. L. Platt, unpublished observations). Although this mechanism might explain the absence of teratomas in out-bred mice, it cannot explain the lack of teratoma formation in highly inbred strains. Moreover, Fandrich et al. (31) found that allogeneic embryonic stem cells may even induce tolerance in rats. Thus, some mechanisms other than cellular immunity must explain the rapid clearance of fetal stem cells from the maternal circulation and the failure of those cells to form teratomas. Our findings that pluripotent embryonic stem cells are exquisitely sensitive to the alternative pathway of complement and that teratomas form rapidly in complement-deficient mice suggest that complement might clear pluripotent fetal cells from the maternal circulation and prevent the formation of teratomas. However, because C3-deficient mice do not appear to experience a high incidence of spontaneous teratomas, some mechanism besides complement must also confer protection. We believe susceptibility to complement may have broader implications than what we have found for embryonic stem cells. We recently advanced the concept that primitive cells can fuse with mature cells and in so doing cause reprogramming and proliferation (32). Susceptibility to complement may prevent these undifferentiated cells from becoming tumors.

Although complement prevents engraftment of embryonic stem cells and tumor formation in some circumstances, it clearly does not pose an insurmountable barrier. We found (as have others) that given in sufficient numbers, embryonic stem cells can form tumors in mice with an intact complement system. Similar observations have been made for lymphoblastoid tumor cells (33–35). These cells are sensitive to complement in vitro but form tumors when introduced in vivo. This apparent discrepancy between in vitro and in vivo susceptibility to complement may have several explanations. First, tumor formation may occur with temporary impairment of complement function. Studying 336 subjects with Sjogren’s syndrome, Ramos-Casals et al. (36) found the development and progression of lymphoma are associated with hypocomplementemia. Second, tumor formation may occur when cells acquire mutations that confer resistance to complement. Sanchez-Perez et al. (37) induced killing of melanoma cells in mice using a DNA vaccine encoding Hsp70 and a suicide gene targeted to melanocytes and found that the melanoma cells remaining after therapy lacked melanin and tyrosinase, two potential Ags to which an immune response had been directed. Tumor cells might similarly be selected for complement resistance. Ajona et al. (38) found that some lung cancer cell lines secrete high levels of Factor H, which controls activation of the alternative pathway of complement. However, we think it noteworthy that tumor formation as we
observed it required administration of at least 1 × 10^5 cells. Because the number of cells administered has an absolute rather than a relative impact on tumor formation, we would postulate that the larger numbers of cells establish a microenvironment, perhaps an aggregate of cells, into which complement penetrates poorly, conducive to the survival of “protected” cells. Once blood vessels invade the mass, the vascular walls may provide a further barrier to complement proteins (39). Preliminary experiments from our laboratory support this hypothesis. We found that an aliquot of 2.5 × 10^6 embryonic stem cells always forms teratomas when introduced as a single dose. However, if the 2.5 × 10^6 cells are divided into eight aliquots and injected separately teratomas do not form.

Our findings may have practical implications for the use of embryonic stem cells and their differentiated progeny for the treatment of disease. Clearly, transplantation of undifferentiated embryonic stem cells might expose patients to the risk of tumor formation. Evans and Kaufman (23) found that undifferentiated embryonic stem cells transplanted s.c. into nude-beige mice form teratocarcinomas. Although the risk of tumor formation should be lower if one transplants cells differentiated from embryonic stem cells, residual undifferentiated embryonic stem cells within the graft might still form tumors; and, our concept of cellular aggregates does not require that all or even most of the transplanted cells be stem cells. Consistent with this explanation, Fujikawa et al. (40) observed formation of teratomas expressing OCT-4, and thus likely arising from undifferentiated embryonic stem cells, in NOD/SCID mice transplanted with pancreatic islet-like clusters that had been derived from embryonic stem cells. Similarly, Sipione et al. (41) observed formation of teratomas in SCID-beige mice transplanted with insulin-expressing cells derived from embryonic stem cells. Our results suggest that propensity of residual undifferentiated cells to form tumors in grafts of differentiated cells might be decreased by treating the grafts with complement before transplantation.

**Disclosures**

The authors have no financial conflict of interest.

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