Hematopoietic progenitor cells grow on 3T3 fibroblast monolayers that overexpress growth arrest-specific gene-6 (GAS6)

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Pluripotential hematopoietic stem cells grow in close association with bone marrow stromal cells, which play a critical role in sustaining hematopoiesis in long-term bone marrow cultures. The mechanisms through which stromal cells act to support pluripotential hematopoietic stem cells are largely unknown. This study demonstrates that growth arrest-specific gene-6 (GAS6) plays an important role in this process. GAS6 is a ligand for the Axl (Ufo/Ark), Sky (Dtk/Tyro3/Rse/Brt/Tif), and Mer (Eyk) family of tyrosine kinase receptors and binds to these receptors via tandem G domains at its C terminus. After translation, GAS6 moves to the lumen of the endoplasmic reticulum, where it is extensively γ-carboxylated. The carboxylation process is vitamin K dependent, and current evidence suggests that GAS6 must be γ-carboxylated to bind and activate any of the cognate tyrosine kinase receptors. Here, we show that expression of GAS6 is highly correlated with the capacity of bone marrow stromal cells to support hematopoiesis in culture. Nonsupportive stromal cell lines express little to no GAS6, whereas supportive cell lines express high levels of GAS6. Transfection of the cDNA encoding GAS6 into 3T3 fibroblasts is sufficient to render this previously nonsupportive cell line capable of supporting long-term hematopoietic cultures. 3T3 cells, genetically engineered to stably express GAS6 (GAS6-3T3), produce a stromal layer that supports the generation of colony-forming units in culture (CFU-c) for up to 6 wk. Hematopoietic support by genetically engineered 3T3 is not vitamin K dependent, and soluble recombinant GAS6 does not substitute for coculturing the hematopoietic progenitors with genetically modified 3T3 cells.

The environment created by the adherent stromal monolayers that develop in long-term bone marrow cultures (LTBMCs) replicates many of the properties of the *in vivo* bone marrow microenvironment. Primary stromal cell cultures derived from LTBMC and immortalized cell lines derived from these cultures support hematopoiesis *in vitro* (1–10). The mechanism through which these stromal cells support hematopoiesis is unclear. Analysis of known stromal cytokines has failed to establish a relationship between cytokine production and hematopoietic support.

We have isolated a series of (murine) bone marrow stromal cell lines that differ greatly in their ability to support hematopoiesis and have used these to try to determine the molecular basis for the supportive phenotype (11). A PCR-driven subtractive hybridization technique was used to prepare a cDNA library enriched for mRNAs differentially expressed by cells that support *in vitro* hematopoiesis. The library was generated by subtracting cDNAs prepared from a nonsupportive stromal line from cDNAs prepared from a supportive line. One of the cDNAs in this subtracted library encoded the message for the product of growth arrest-specific gene-6 (GAS6). GAS6 was initially characterized as a gene whose expression was up-regulated in serum-starved NIH 3T3 fibroblasts (12). Not all 3T3 lines express GAS6, and it is not expressed by the cells used in our experiments. It is a ligand for the Axl (Ufo/Ark), Sky (Dtk/Tyro3/Rse/Brt/Tif), and Mer (Eyk) families of tyrosine kinase receptors and binds to these receptors via tandem G domains at its C terminus (13, 14). The production of GAS6 with full biological activity requires a vitamin K-dependent posttranslational modification in which the newly synthesized peptide is extensively γ-carboxylated in the endoplasmic reticulum. GAS6 is ubiquitously expressed and has been implicated as a mitogen for endothelial cells (15), neural cells (16), and vascular smooth muscle cells (17), as well as a factor capable of inducing cell adhesion (18) and chemotaxis (19). Although it is expressed highly in the bone marrow, its role in hematopoiesis is unknown (20).

In this paper, we show that 3T3 fibroblasts, genetically engineered to overexpress GAS6, support hematopoiesis in culture far better than unmodified 3T3 or 3T3 cells transfected with the plasmid vector alone. Recombinant soluble GAS6, added to cultures of unmodified 3T3, does not reproduce the hematopoietic effects of the genetically engineered cells. Hematopoietic support by stromal cells expressing GAS6 is not vitamin K dependent.

Materials and Methods

Cell Lines. The supportive and nonsupportive stromal cell lines were derived from BALB/c LTBMCs. Their isolation and properties have been described elsewhere (11). NIH 3T3, a cell line known to be incapable of long-term hematopoietic support (21), was used as a negative control, and MS-5 murine stromal cells (22) were included in some experiments as a positive control. The 3T3 line, which does not produce GAS6, was a gift of Claudio Basilico (New York University School of Medicine).

Media. Stromal cells were cultured in complete Iscove's medium (IDMEM; GIBCO/BRL). Transfected 3T3 cells were cultured in IDMEM supplemented with G418 (800 μg/ml). Assays for hematopoietic activity were conducted with LTBMC medium, Myelocult M5300 (alpha MEM containing 12.5% equine serum, 12.5% FBS, 0.2 mM i-inositol, 20 mM folic acid, 10^{-4} M β-mercaptoethanol, 2 mM L-glutamine, and 10^{-6} M hydrocortisone; StemCell Technologies; Vancouver).

GAS6 cDNA and Recombinant GAS6 (rGAS6). Both murine GAS6 cDNA and the recombinant protein were supplied by Paola Bellosta in the laboratory of Claudio Basilico at the New York University School of Medicine (23). The cDNA was supplied as an *Eco*RI insert in the plasmid pRK5. rGAS6 was obtained from GAS6-transfected Chinese hamster ovary cells grown in serum-free DMEM containing 5 μg/ml insulin, 5 μg/ml transferrin, 10 mM proline, and 4 μM menadione. The rGAS6 was purified by using a hydroxyapatite column. The product obtained from the column gives a single band on PAGE and stimulates the growth of mesenchymal cell lines (24). The concentration of active protein was

Abbreviations: GAS6, growth arrest-specific gene-6; rGAS6, recombinant GAS6; LTBMC, long-term bone marrow culture; HRP, horseradish peroxidase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CFU-c, colony-forming units in culture.

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determined by binding to the murine Axl-Fc fusion protein by using BIACORE and by the ability to phosphorylate the Axl receptor(25).

Anti-GAS6 Antibodies. Two polyclonal rabbit anti-mouse GAS6 antibodies were also provided by P. Bellosta. Antibody 570 was raised against the sequence Ala-Ser-Tyr-Lys-His-Ser-Asp-Ile-Thr-Ser-His-Ser-Cys-Pro near the C terminus of GAS6. Antibody 559 was raised against the sequence Pro-Asp-Gln-Cys-Thr-Pro-Asn-Pro-Cys-Asp-Lys-Lys between amino acids 114 and 125 of the 673-aa protein. The antibodies (diluted 1:50) were adsorbed with confluent 3T3 cells that had been transfected with pcDNA 3.1 vector without an insert at 25°C for 1 h before use as immunofluorescence reagents.

PCR-Driven Subtractive Hybridization. GAS6 was identified through a PCR-based subtractive hybridization technique (26, 27) (PCR-Select; CLONTECH). cDNA was prepared from 100 ng of poly(A)$^+$ mRNA for the "tester" cells (stromal line D5.2-96, a line that supported extensive hematopoiesis in culture) and 300 ng of mRNA from the "driver" (subtracting) cells (stromal line B6.1, a nonsupportive line). Both cDNAs were digested with *Rsa*I. The tester cDNA was divided into two portions, and each was ligated to one of the two adaptors in the kit. The driver DNA was not ligated. The tester cDNAs and the driver cDNA were denatured, and the driver was hybridized with each of the tester samples for 8 h at 68°C. This hybridization enriches low abundance sequences in the residual single-stranded material. The two hybridization mixtures (one for each of the adaptors) were mixed together *without being denatured again.* Additional denatured driver was added, and the mixture was hybridized for 16 h at 68°C. Differentially expressed cDNAs were then amplified by PCR. The missing complementary strands of the adaptors were filled in by a brief incubation at 75°C. and then thermally denatured to begin a PCR cycle. After 30 cycles, the reaction was stopped, and a small aliquot was removed, diluted, and amplified further by using nested primers to produce the differentially enriched cDNAs. Abundant and rare mRNAs are normalized in the process.

Cloning of PCR-Amplified cDNAs. The difference product was electrophoresed through a 1.3% agarose gel. Three discrete bands were visible after ethidium bromide staining. These bands were excised, the DNA extracted (Qiaquick gel extraction kit; Qiagen, Chatsworth, CA) and ligated into pCR2.1 (Invitrogen). Competent cells of *Escherichia coli* (InvF) were transformed and plated. Ten colonies per band were isolated and expanded in LB medium containing 50 μg/ml ampicillin. The plasmids were isolated via alkaline lysis and sequenced. Sequences of subtracted cDNAs were compared with those in the public nucleotide databases to identify sequence homologies, by using the BLAST program at the National Center for Biotechnology Information internet web site (www.ncbi.nlm.nih.gov).

Northern Blotting. Stromal mRNA (5 μg) or 30 μg of total RNA was denatured and run for 3 h at 50 mV in a 1.2% agarose, 6.6% formaldehyde, 1× Mops containing gel. mRNA was transferred to nitrocellulose (GIBCO/BRL) and baked. The complete GAS6 ORF cDNA, radiolabeled with [α-^{32}P]dCTP via random priming (Prime-a-gene; Promega) was used as a probe.

Transfection of Murine GAS6 into NIH 3T3 Fibroblasts. Full-length cDNA was cloned into pcDNA3.1(+) (Invitrogen) by using lipofectamine (GIBCO/BRL) following the distributor's protocol. Clones of G418-resistant cells were isolated under limit-dilution conditions. Total RNA was collected from nine clones to monitor the expression of GAS6. mRNA from the two lines expressing the highest levels was used for Northern analysis.

Western Blotting. Cells (1.5- to 2.0 × 10^6) for each cell line were lysed with SDS/PAGE loading buffer and denatured at 100°C for 5 min. Samples were electrophoresed through a 12.5% SDS gel, transferred to a nitrocellulose membrane, and stained with a (primary) polyclonal rabbit anti-mouse GAS6 antibody. The membrane was then stained with a secondary horseradish peroxidase (HRP)-labeled conjugate (donkey anti-rabbit; Affinity BioReagents, Golden, CO), washed, and exposed to a detection mixture containing H_2O_2, phenol, and luminol. The light emitted by the HRP/H_2O_2-catalyzed oxidation of luminol was detected by a short exposure to blue light-sensitive autoradiography film.

Cytokine Expression by GAS6-Transfected 3T3. A multiprobe ribonuclease protection assay (RPA) system was used to analyze cytokine and growth factor expression by parental and GAS6-transfected cell lines. Antisense probes hybridizing with tumor necrosis factor (TNF)-β, lymphotoxin (LT), TNF-α, IL-6, IFN-γ, IFN-β, transforming growth factor (TGF)-α1 and -2, granulocyte–macrophage colony-stimulating factor (GM-CSF), G-CSF, macrophage inflammatory protein (MIP)-1α, flt-3 ligand (FL), thrombopoietin (TPO), lymphocyte inhibitory factor (LIF), IL-3, IL-7, stem cell factor (SCF), M-CSF, and pre-B cell growth-stimulating factor (PBSF) [stromal cell-derived factor (SDF)-1] were prepared.

With the exception of probes used to identify inflammatory cytokine messages, which were obtained commercially (PharMingen; probe set mCK-3), the probes were synthesized by using published sequences. RNA was isolated from mouse (BALB/c) liver, kidney, spleen, and bone marrow and pooled. Reverse transcription-PCR was used to create cDNA from the pooled mRNA. The G-CSF probe was prepared from interferon-stimulated peritoneal cells. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a reference marker. The PCR products were ligated into a plasmid vector (pCR3-Uni) that contains the T7 promoter. Primers were chosen so that the amplified sequences would differ from each other by 5–15 bp. These differences in probe length allowed templates to be pooled into sets. In each probe set, the housekeeping gene GAPDH was used to standardize and quantify the samples. T7 RNA polymerase was used to transcribe [α-^{32}P]UTP-labeled probes from the plasmid mixtures. Total RNA was prepared from both cell lines, and 30 μg from each was used for hybridization. After hybridization, the samples were digested with ribonucleases A and T1, and the protected fragments were separated on a sequencing polyacrylamide gel. A probe for GAPDH was included in each set, and all samples contained similar amounts of mRNA. The protected fragments were identified by phosphorimaging.

***In Situ* Immunostaining of Transfected Cell Lines.** All antibodies were diluted 1:100 before staining. Cells were grown to ≈75% of confluence on 2-well chamber slides. One well was stained with antibody 570 (anti-GAS6) washed 3 times with 1× PBS and stained with FITC-conjugated antibody (goat F(ab′)$_2$ anti-rabbit IgG [H + L] human adsorbed; Caltag, South San Francisco, CA). The second well was stained with normal rabbit serum as a control. After staining, the slides were washed and fixed with 4% para-formaldehyde.

FACS Analysis of Stromal Cell Lines. Cells (2 × 10^5) were incubated with anti-GAS6 antibody at a 1:50 dilution for 30 min at 4°C, washed twice with PBS, and stained with goat anti-rabbit IgG. They were washed and fixed in 4% para-formaldehyde. Controls were stained with 3T3-absorbed normal rabbit serum. The staining was evaluated by using a FACScan (Becton Dickinson) flow cytometer.

Evaluation of Hematopoietic Support. Monolayers of GAS6-transfected NIH 3T3, vector-transfected NIH 3T3, and NIH 3T3 were grown to near confluence in 12-well plates and irradiated

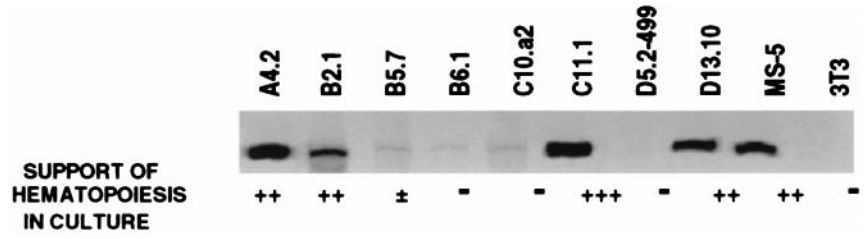

Fig. 1. Ribonuclease protection assay showing the expression of GAS6 mRNA by supportive and nonsupportive cell lines. Thirty micrograms of total RNA from each clone was hybridized to a radiolabeled antisense GAS6 probe. The capacity of these lines to support hematopoiesis was described in Dormady et al. (11). Line D5.2-499 is a nonsupportive clone that evolved from the previously supportive D5.2 line.

(12,500 cGy). Low-density bone marrow cells (40,000) were added to each well and cultured at 33°C for up to 50 days. Each week, one half of the medium, along with the equivalent proportion of nonadherent cells, was removed from the cultures and replaced with fresh medium. At various time points (e.g., days 7, 14, 21, 28, and 35), progenitor cells (adherent and nonadherent) were collected and cultured in complete methylcellulose (Methocult M3434; StemCell Technologies) at 37°C for 7–10 days, and myeloid colonies were counted. Two or three replicate wells were assayed in duplicate at each time point. In some experiments, both adherent and nonadherent progenitors were assayed.

Results

Stromal Cell Lines That Support Hematopoiesis in Culture Express GAS6. PCR-driven subtractive hybridization was used to identify mRNAs differentially expressed by stromal cell lines that support murine hematopoiesis in culture. After subtraction, differentially expressed cDNAs were amplified, and the PCR products were isolated and cloned. Inserts from 17 clones were sequenced. Four of these corresponded to GAS6. Other genes identified in the subtracted library include murine SDF-5 (stromal-derived factor-5), human lymphoid nuclear protein, murine CCAAT/enhancer binding protein, murine lipoprotein lipase, and murine C3 (complement component) (Data not shown).

If GAS6 is required for the support of hematopoiesis in culture, all supportive lines should express this protein. We examined the relationship between GAS6 expression and hematopoietic support in bone marrow-derived cell lines (11). GAS6 was expressed strongly in primary murine bone marrow stromal cells and 5/5 cell lines that could support hematopoiesis in culture. It was expressed weakly or not at all in 5/5 nonsupportive cell lines (Fig. 1. C11.1, our most supportive cell line, expressed the highest level of GAS6. NIH 3T3, a cell line known to be nonsupportive, expressed no GAS6. The result indicates a correlation between the expression of GAS6 and stromal support of hematopoiesis. When first isolated, the stromal cell line designated D5.2 was highly supportive of hematopoiesis in culture and expressed high levels of GAS6. After continuous passage in culture, this line lost its ability to support *in vitro* hematopoiesis. The line was recloned in an unsuccessful attempt to recover a line with the supportive phenotype. The newly derived clone (D5.2-499) neither supported hematopoiesis nor expressed GAS6.

3T3 Fibroblast Monolayers That Overexpress GAS6 Support Hematopoiesis in Culture. To test the correlation between the expression of GAS6 and stromal support of hematopoiesis, we attempt to engineer a supportive phenotype by transfecting GAS6 cDNA into a nonsupportive cell line. NIH 3T3 was used because it neither supports hematopoiesis nor, under normal conditions, expresses GAS6 (21). GAS6 was not detected in parental 3T3 or vector-transfected 3T3 (neo-3T3)(Fig. 2*a*). In the transfected cells, a 2.5-kb GAS6 transcript was readily detectable. GAS6-3T3-G3 expressed GAS6 mRNA at a level comparable to that of the highly supportive C11.1. The protein product was detectable by Western blotting of cell lysates of the transfectants (Fig. 2*b*). GAS6-3T3-G3 produced more GAS6 protein than did the sibling G6 clone, and in fact the level produced by the transfectant was comparable to that produced by the stromal cell line C11.1. The full-length molecule was also detected in culture supernatants obtained from the transfectants (Fig. 2*c*.)

At all times tested (7–35 days of culture), BMC cultured on GAS6-transfected 3T3 produced more CFU in culture (CFU-c) than did BMC-cultured on NIH 3T3 or 3T3 transfected with vector alone (neo-3T3) (Fig. 3*a*) The effect of transfection with GAS6 is

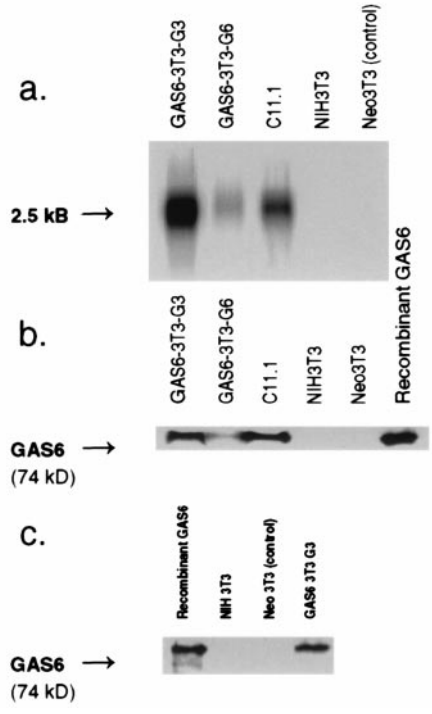

Fig. 2. Expression of GAS6 by transfected cell lines. (*a*) Expression of mRNA (Northern blot). Full-length GAS6 cDNA was radiolabeled and used as a probe to determine the level of GAS6 mRNA expression by GAS6-3T3-G3, GAS6-3T3-G6, C11.1, NIH 3T3, and vector-transfected 3T3 ("control"). GAPDH mRNA was also measured to assure that equal amounts of RNA had been applied to each lane. (*b*) Expression of GAS6 protein by 3T3-GAS6 (Western Blot). GAS6-3T3-G3, GAS6-3T3-G6, C11.1, NIH 3T3, and vector-transfected 3T3 were lysed in SDS/PAGE loading buffer and electrophoresed. After transfer, the nitrocellulose membrane was stained with polyclonal rabbit anti-mouse GAS-6 antibody, and the transferred proteins were visualized with donkey anti-rabbit IgG-HRP. HRP was detected via chemiluminescence by using luminol as the substrate. The first lane (far left) contained 150 ng of purified rGAS6 as a control. (*c*) GAS6 is secreted by GAS6-3T3-G3. Supernatants from GAS6-3T3-G3, NIH 3T3, and control 3T3 cultures were collected and concentrated 5-fold via centrifugation through a Millipore protein spin column. The concentrated culture medium was analyzed as described in Fig. 2*b*.

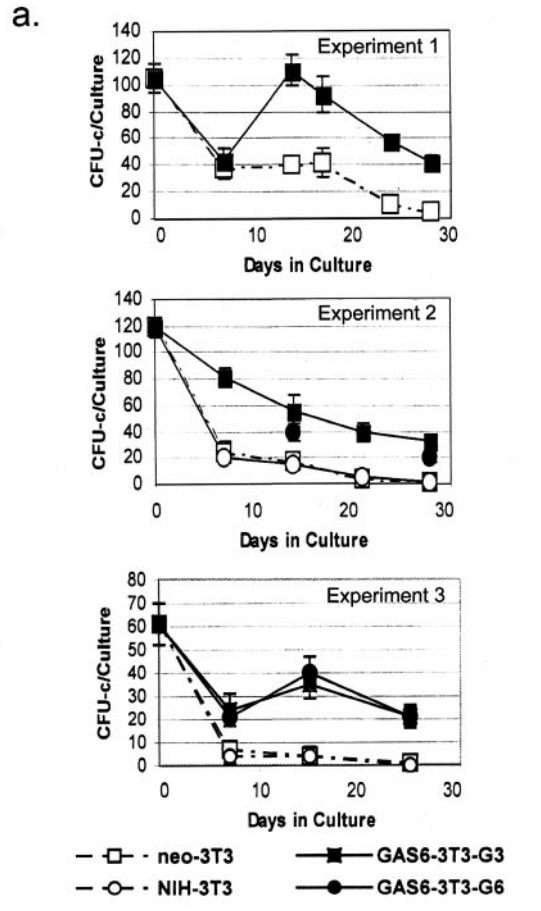

Fig. 3. Hematopoietic support by GAS6-transfected 3T3 Cells. (*a*) CFU-c in cultures supported by GAS6-transfected 3T3. Results from three representative experiments are shown. The results presented are for total CFU-c per 2.0 ml culture and are uncorrected for the loss of nonadherent CFU-c at each feeding. The values shown are mean ± SD ($n = 4–6$). (*b*) Nonadherent cells per culture. The results from a single experiment (Exp. 3 above) are shown. The values shown are mean ± SD ($n = 6$) and are uncorrected for the consequences of demi-depletion at each weekly feeding.

not on the number hematopoietic cells produced in the cultures. Both the vector-transfected and GAS6-transfected cells produced similar numbers of hematopoietic cells (mostly granulocytes), but only cultures on GAS6-transfected 3T3 generated hematopoietic progenitors (Fig. 3 *a* and *b*). To ensure that the supportive phenotype was not an artifact of the transfection process, we tested the supportive capacity of a second transfected 3T3 clone. Again, the GAS6-transfected clone (GAS6-3T3-G6) was more supportive

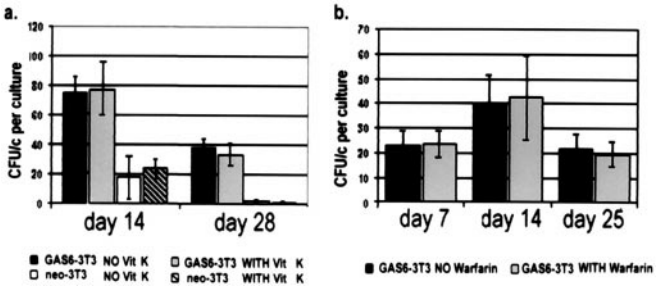

Fig. 4. Hematopoietic support by GAS6-transfected 3T3 is not vitamin K dependent. (*a*) Effect of additional vitamin K on hematopoietic support by GAS6-3T3. (*b*) Effect of warfarin on hematopoietic support by GAS6-3T3. Vitamin K (4 μM menadione) and warfarin (1 μM) were present throughout the culture period and were added to the medium used to feed the cultures. Nonadherent CFU-c were assayed at the indicated times. Hematopoietic colonies were counted after 7–10 days in culture. The error bars indicate the standard deviation of the results ($n = 4–6$).

than its paired, vector-transfected control (Exps. 2 and 3 of Fig. 3a). The three experiments shown in Fig. 3*a* illustrate the variability in both the magnitude of the response and the kinetics of CFU-c production in these cultures. The results shown in the figures underestimate the extent of proliferation of the CFU-c in the cultures because they have not been adjusted for the effect of removing half of the accumulated nonadherent cells at the time of each weekly feeding.

Vitamin K Is Not Required for Hematopoietic Support by GAS6-3T3. Because the media used to show hematopoietic support by GAS6-transfected cells did not contain added vitamin K, the results shown in Fig. 4 suggest that the molecule active in hematopoiesis is not carboxylated. To clarify the role of carboxylation, the hematopoietic support provided by GAS6-3T3 cells was measured in the presence of added vitamin K (4 μM menadione) and in the presence of warfarin (1 μM), an inhibitor of vitamin K-dependent carboxylation. The results are shown in Fig. 4 *a* and *b*. Neither treatment altered the production of CFU-c.

Soluble GAS6 Does Not Support Hematopoiesis. Attempts to reproduce the supportive phenotype by supplying purified exogenous rGAS6 in long-term culture systems were unsuccessful. Mouse BMC cultured on irradiated 3T3 monolayers in the presence of concentrations of rGAS6 ranging from 1 ng/ml to 1 μg/ml were indistinguishable from cultures without GAS6. rGAS6 also failed to produce a supportive phenotype when added to cultures of the nonsupportive stromal clones D5.2 and C10a.2. Soluble rGAS6 also adversely effects primary LTBMCs. When added to cultures in which low-density nonadherent mouse BMC were cultured on a supportive stromal layer (stromal clone C11.1), both CFU-c adhering to the stromal layer (adherent) and CFU-c released into the culture medium (nonadherent) were significantly reduced (day 20/nonadherent/$P < 0.01$; day 20/adherent/$P < 0.05$; day 30/nonadherent/$P < 0.005$; day 30/adherent/$P < 0.05$) (Fig. 5*a*). rGAS6 added to primary cultures of mouse bone marrow also inhibited the production of myeloid progenitors (Fig. 5*b*). By 12 days, bone marrow cultured without GAS6 had become actively hematopoietic. In contrast, in cultures grown in the presence of GAS6 the stromal monolayers grew rapidly but were minimally hematopoietic. By day 24, stromal monolayers with obvious hematopoiesis had developed in all cultures, but the monolayers in the GAS6-supplemented cultures had fewer hematopoietic foci and these were small and less well developed (data not shown).

Purified rGAS6 also inhibits the growth of hematopoietic colonies (CFU-c) in semisolid medium supplemented with optimum amounts of recombinant growth factors. There was no difference

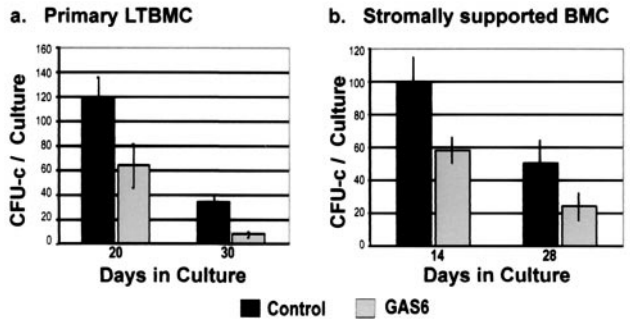

Fig. 5. GAS6 inhibits LTBMC. (*a*) Effect on primary LTBMC. Total BMC (10^7) were cultured with and without rGAS6 (1.0 μg/ml). Nonadherent CFUs were assayed 20 and 30 days after the initiation of the cultures. The error bars indicate the standard deviation of the results ($n = 6$). (*b*) Effect on stromally supported cultures. Low density BMC (5×10^4) were added to a confluent monolayer of irradiated (20,000 cGy) C11.1 stromal cells. rGAS6 (1.0 μg/ml) was added to half of the cultures. Nonadherent CFU-c were assayed 2 and 4 wk after the initiation of the cultures. The error bars indicate the standard deviation of the results ($n = 6$)

between the number of colonies formed by cells plated without GAS6 and cells plated in 100 ng/ml GAS6 (93.3 ± 4.5 vs. 92.3 ± 3.6). However, at 1 μg/ml, GAS6 inhibited colony formation (45.3 ± 5.5, $P < 0.0005$). In a similar experiment, low-density bone marrow cells were plated in recombinant growth factor-deficient methylcellulose medium and recombinant growth factor-deficient methylcellulose supplemented with GAS6 to see whether GAS6 alone promoted colony formation. The cultures were examined weekly for 3 wk. No colonies of any type grew.

The Pattern of Hematopoietic Cytokine Expression Is Not Altered in GAS6-3T3 Cells. Because rGAS6 was unable to support CFU-c production in long-term cultures, we examined the possibility that GAS6-transfected cells either produced new cytokines or growth factors or were induced to secrete different amounts of factors that were part of their normal repertoire. By using a ribonuclease protection assay, we compared the expression of steady state levels of mRNA for 18 cytokines and growth factors that play a role in the regulation of hematopoiesis by control and GAS6-transfected 3T3 cells. No significant changes were observed (data not shown). We also tested the effect of medium conditioned by the growth of GAS6-3T3-G3 on the capacity of NIH 3T3 cells to support hematopoiesis. The GAS6-3T3 cells were grown in LTBMC medium. At confluence, the medium was removed, filtered, and used to feed NIH 3T3 cells. After 48 h, the cells were irradiated, and 1.0×10^5 low density BMC were added. No effect on hematopoiesis was seen (data not shown).

GAS6 Is Associated with the Surface of GAS6-Transfected Cells. GAS6 was readily detected in lysates of adherent GAS6-3T3 cells (Fig. 2*b*). If this cell-associated GAS6 was on the surface of the cells, it could serve as a membrane-bound ligand and have different properties than the molecule in solution. Immunofluorescence was used to identify GAS6 on the cell surface. Unfixed cells were stained, either *in situ*, on the surface of plastic slides, or after removal from the culture vessels, by using flow cytometry. Fig. 6 shows that GAS6-3T3 cells can be stained with polyclonal anti-GAS6 peptide antibodies. Many, but not all of the cells were stained by this antibody. The staining was dim, but the long processes that extend from the cells were clearly visualized. The specificity of the staining was confirmed by flow cytometry. GAS6-3T3 cells stain more brightly with anti-GAS6 than do control cells (Fig. 6 C, parts 1 and 2) and the staining can be inhibited by soluble rGAS6 (Fig. 6 C, part 3).

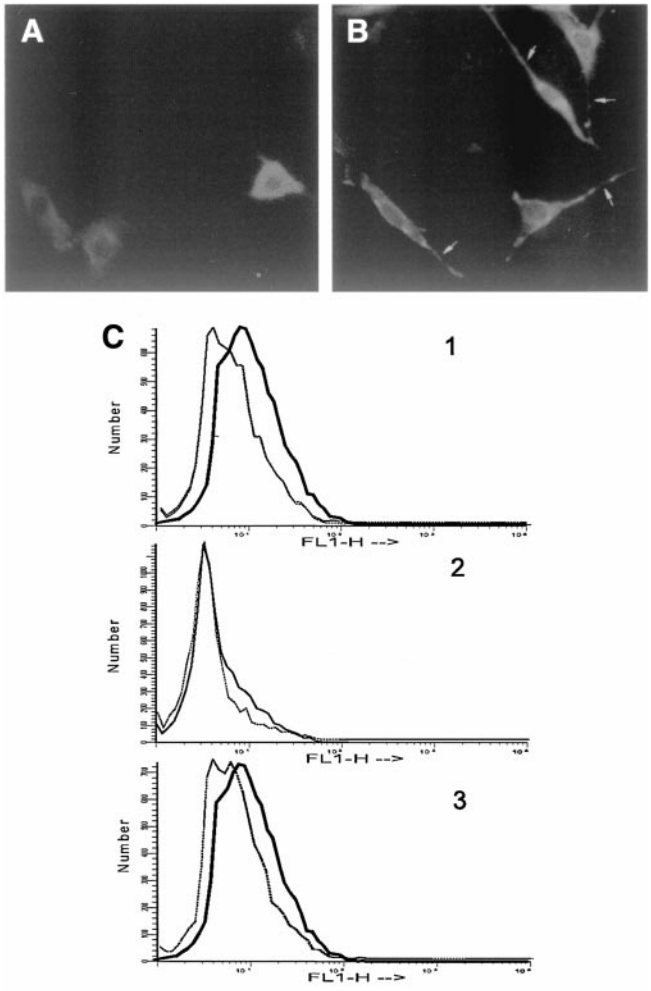

Fig. 6. GAS6-transfected cells stain for surface-associated GAS6. (*A* and *B*) Indirect immunofluorescent staining with anti GAS6 antibodies. The cells were grown on glass chamber slides and stained without fixation to avoid permeabilization of the cell membranes (original magnification ×600). (*A*) neo3T3 stained with polyclonal rabbit anti-GAS6 peptides (no. 559). Many cells show nonspecific fluorescence associated with cell body. No membrane staining was seen, and the cell processes were not stained. (*B*) GAS6-3T3 cells stained as above. Note the bright staining of the long processes of this fibroblast (indicated by arrows in the figure) as well as the increased staining of the cell membrane around the cell bodies. (*C*) Flow cytometric demonstration of GAS6 expression of the surface of transfected 3T3 cells. Histograms of the fluorescence intensity after staining GAS6-3T3 and neo-3T3 with rabbit anti GAS6 (no. 559). Five thousand cells were counted in list mode. The data were analyzed after electronic gating to exclude dead cells. Part 1, Anti-GAS6 stains GAS6-3T3-G3. Solid line, Anti-GAS6; dashed line, normal rabbit serum. Part 2, Anti-GAS6 does not stain neo-3T3. Solid line, Anti-GAS6; dashed line, normal rabbit serum. Part 3, Soluble recombinant GAS6 inhibits the staining of GAS6-3T3-G3. Solid line, Anti-GAS6; dashed line, anti-GAS6 plus rGAS6

Discussion

Expression of GAS6 by stromal cell lines correlates with the capacity of the stromal cells to support *in vitro* hematopoiesis. Overexpression of GAS6 by nonsupportive mesenchymal cells (3T3) allows these cells to support long-term hematopoiesis in culture. Stable transfection of only one gene into the nonsupportive cells created a supportive phenotype. The mRNA transcript found in the transfected cells was of the expected (2.5 kb) size for GAS6, indicating that no unusual splicing event had occurred. Both the material located in the cellular monolayer and the secreted material had a mass of 74 kDa, demonstrating that the predominant product was full-length GAS6.

The mechanism by which overexpression of GAS6 (by 3T3 cells) stimulates hematopoiesis in culture is not clear. GAS6 is both a mitogen and a survival factor for mesenchymal cells (15, 23) and may act to preserve the stromal monolayer. Although GAS6 preserved stromal integrity in irradiated cultures and viability in primary bone marrow cultures (data not shown), rGAS6 did not alter the supportive capacity of NIH 3T3 or other nonsupportive cell lines. It is thus unlikely that GAS6 maintains hematopoiesis by preserving the stromal layer. It also seems unlikely that transfected GAS6 acts indirectly by stimulating new cytokine production in the monolayer because 3T3 grown in GAS6-3T3 conditioned medium did not support hematopoiesis. We sought but did not find evidence for autocrine or paracrine effects of GAS6 in the stromal layer. The steady state levels of mRNAs encoding 18 cytokines and growth factors that are known to affect hematopoiesis were the same in transfected and untransfected cells.

The difference in the effect of material produced by the transfected cells and soluble rGAS6 is unexplained. It could be due to the manner in which the molecules are presented to the responding cells. IFN-γ expressed by stromal cells in LTBMC is far more inhibitory than exogenously provided recombinant IFN (28). Selleri *et al.* postulated that either local effects of the product on progenitor cells that were in intimate contact with the stromal layer or indirect effects leading to an alteration in feeder function were responsible for the phenomenon. Local microenvironmental effects could explain the activity of GAS6, because membrane-associated GAS6 could act on progenitors adherent to the stromal cells or it could serve as a ligand to promote cell–cell contact between stem/progenitor cells and stromal cells. Alternatively, GAS6 may act as a chemoattractant (19). Hematopoietic cells of all lineages express Axl (29, 30), and GAS6 produced by stromal cells could induce chemotaxis of Axl-expressing progenitor cells.

It is possible that a membrane-associated form of GAS6 is responsible for its hematopoietic effects and that membrane association is the critical factor required to render GAS6 active. The immunofluorescence data indicate that some GAS6 associates with the cell membrane, but the nature of the association is unknown. GAS6 lacks both the hydrophobic transmembrane domains required for membrane anchoring and the structural requirements necessary for phosphatidylinositol-mediated membrane linkage. GAS6 synthesized in the presence of vitamin K can bind to phosphatidylserine in the plasma membrane via its γ-carboxyglutamic acid residues (31), but the uncarboxylated form produced by the cells in our experiments should not be capable of this interaction. An uncarboxylated form of GAS6 could be secreted and bind to proteoglycans on the surface of stromal cells. Alternatively, the surface-associated GAS6 could be receptor bound. It could be associated with the membrane via its known receptors, Axl, Sky, and Mer, which are expressed on mesenchymal cells, or with a novel receptor that recognizes the epidermal growth factor-like domains. Bound in this way, GAS6 could mediate either chemotaxis or cell–cell interactions. Another (albeit remote) possibility is that GAS6 could remain anchored to the membrane-bound carboxylase within the endoplasmic reticulum. The GAS6–carboxylase complex might pass through the Golgi and appear on the cell surface. Whatever the mechanism of attachment to the cell surface, it appears that membrane-associated GAS6 plays a critical role in mediating its activity in hematopoietic cultures. The ratio of membrane-associated and free GAS6 may determine the fate of hematopoietic cells in culture and perhaps in the intact organism.

Among the intriguing aspects of this GAS6-mediated hematopoietic support is its independence of vitamin K. The addition of vitamin K to the culture medium does not improve support, and GAS6-3T3 cells grown in the presence of warfarin, which inhibits vitamin K-dependant carboxylation, are as supportive as those grown in vitamin K-containing medium. These results demonstrate that the supportive phenotype does not require carboxylation of the molecule. GAS6 *synthesis* does not require vitamin K; it proceeds in the presence of warfarin but the product synthesized is uncarboxylated and does not activate its cognate receptor(s). GAS6 contains an NH2-terminal Gla domain followed by four epidermal growth factor-like repeats and tandem globular (G) domains(14). The G domains are responsible for binding to the TK receptors. Mutated GAS6, lacking either the Gla domains and/or the epidermal growth factor-like repeats, activates the cognate tyrosine kinase receptors. Proteolytic cleavage of the uncarboxylated, wild-type molecule, removes the uncarboxylated Gla region and converts the inactive form to one that can activate ARK family receptors but remains unable to stimulate the proliferation of vascular smooth muscle cells (32).

It is possible that the transfected 3T3 cells secrete a unique noncarboxylated or posttranslationally modified product that has a different range of activities than carboxylated GAS6. The level of γ-carboxylation could serve as a switch, permitting different functions of the same protein to be regulated. Identification of this alternative product could provide a means of expanding hematopoietic progenitors in culture. If the noncarboxylated form of GAS6 has a biological activity different from the conventional form, it suggests the possibility that the uncarboxylated form of other vitamin K-dependent proteins might also have additional functions.

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