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The Positive Regulatory Effect of TGF-β2 on Primitive Murine Hemopoietic Stem and Progenitor Cells Is Dependent on Age, Genetic Background, and Serum Factors

Els Henckaerts, Jessica C. Langer, Jonathan Orenstein, and Hans-Willem Snoeck

TGF-β is considered a negative regulator of hemopoietic stem and progenitor cells. We have previously shown that one TGF-β isoform, TGF-β2, is, in fact, a positive regulator of murine hemopoietic stem cell function in vivo. In vitro, TGF-β2, but not TGF-β1 and TGF-β3, had a biphasic dose response on the proliferation of purified lin-Scal-/-kit+ (LSK) cells, with a stimulatory effect at low concentrations, which was subject to mouse strain-dependent variation. In this study we report that the stimulatory effect of TGF-β2 on the proliferation of LSK cells increases with age and after replicative stress in C57BL/6, but not in DBA/2, mice. The age-related changes in the TGF-β2 effect correlated with life span in B6D recombinant strains. The stimulatory effect of TGF-β2 on the proliferation of LSK cells requires one or more nonprotein, low m.w. factors present in fetal calf and mouse sera. The activity of this factor(s) in mouse serum increases with age. Taken together, our data suggest a role for TGF-β2 and as yet unknown serum factors in the aging of the hemopoietic stem cell compartment and possibly in organismal aging. The Journal of Immunology, 2004, 173: 2486–2493.

Hemopoietic stem cells (HSC) are capable of self renewal and differentiation into all lineages of mature blood cells. It is still unclear how pool size, renewal, and differentiation of HSC are regulated in vivo. These functional characteristics are subject to quantitative genetic variation in inbred mouse strains. Quantitative trait analysis is therefore an attractive approach to elucidate regulatory mechanisms for HSC in vivo. We have previously demonstrated genetically determined variation in the proliferation of lin-Scal-/-kit+ (LSK) primitive hemopoietic progenitor cells in response to TGF-β2 (11). The dose response of TGF-β2 on the growth of LSK cells supported by early acting cytokines was biphasic, with a stimulatory effect at low concentrations and an inhibitory effect at higher concentrations. This dose response was subject to extensive mouse strain-dependent variation (11). In contrast, and in accordance with previous observations (12–15), the dose responses of TGF-β1 and TGF-β3 were inhibitory and did not show mouse strain-dependent variation, although all isoforms of TGF-β share significant sequence homology and signal through the same receptor complex (16, 17). A quantitative trait locus (QTL) for the effect of TGF-β2 was identified on chromosome 4 overlapping with a QTL regulating the frequency of LSK cells (9), suggesting that TGF-β2 is a regulator of LSK cells in vivo (11). Studies in knockout mice revealed that the stimulatory effect of TGF-β2 on the proliferation of LSK cells, observed at low TGF-β2 concentrations in vitro, is relevant in vivo. The frequency of LSK cells, their proliferative capacity in vitro, as well as the cycling activity and the serial repopulating capacity of HSC were lower in adult Tgfb2−/− mice than in wild-type (wt) littermates. In contrast, no such phenotypes were observed in LSK cells from fetal liver, except after serial transplantation (11).

The more rapid decline of the repopulation capacity of Tgfb2−/− HSC compared with wt HSC after serial transplantation and the presence of a clear hemopoietic phenotype in adult, but not in fetal, TGF-β2-deficient mice (11) suggested a role for TGF-β2 in the regulation of HSC that have undergone replicative or transplant-related stress (18–20). HSC age, as indicated by their lower proliferative capacity in vitro (21) and by the mouse strain-dependent, age-related changes in the number of primitive progenitor and stem cells and in their repopulation capacity in vivo (22–25). Furthermore, the differentiation potential of the HSC compartment appears to become skewed toward the myeloid lineage with age (26–28). As HSC have been shown to cycle (29), replicative stress, even in the absence of detectable telomere erosion (30, 31), may underlie at least some of the age-related changes in HSC (32). Many traits affecting the hemopoietic stem and progenitor cell compartments also change with age in a mouse strain-dependent fashion (21–23, 32–34) and have been implicated in organismal life span (21, 32–34). The responsiveness of LSK cells to TGF-β2 showed mouse strain-dependent variation in young mice. Furthermore, our previous data suggest that TGF-β2 signaling may play a role in the regulation of HSC that have undergone stress (11). Therefore, we examined whether the responsiveness of LSK cells to TGF-β2 changes with age and with hemopoietic stress, and whether these changes show quantitative genetic variation. We show in this study that the stimulatory effect of TGF-β2 increases in a mouse strain-dependent fashion with age and after repeated administration of the myelotoxic drug, 5-fluorouracil (5-FU). The
extent of this increase correlates significantly with life span in BXD recombinant inbred mice. Furthermore, the isoform-specific stimulatory effect of TGF-β2 on the proliferation of LSK cells requires the presence of one or more heat-stable, proteinase K-resistant, low m.w. serum factors. Finally, the biological activity of mouse serum as a modulator of TGF-β2 signaling increases with age. Taken together, our data suggest that TGF-β2 signaling plays a role in the aging of hematopoietic stem and progenitor cell compartments and perhaps in longevity in BXD mice.

Materials and Methods

Mice

Eight-week-old C57BL/6, DBA/2, BALB/c, and BXD recombinant inbred (RI) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Eighteen-month-old DBA/2 and C57BL/6 mice were obtained from the National Institute on Aging or were raised in our animal facility under specific pathogen-free conditions. BXD RI mice were raised to 18 mo of age in our facility. Experiments and animal care were performed in accordance with the Mount Sinai institutional animal care and use committee.

Abs and cytokines

Unconjugated CD2, CD3, CD8, CD4, B220, Lyg/G1, and Mac1, PE-conjugated Scal, CyChrome-conjugated streptavidin, and FITC-conjugated goat anti-rat Abs were purchased from Southern Biotechnology Associates (Birmingham, AL). Unconjugated Ter119 and biotinylated anti-c-Kit were purchased from BD Pharmingen (San Diego, CA). Recombinant mouse Fl3 ligand (Fl3L), Kit ligand (KL), thrombopoietin (TPO), TGF-β1, TGF-β2, polyvalent pan-anti-TGF-β, and monoclonal anti-TGF-β Abs were purchased from R&D Systems (Minneapolis, MN). Affinity-purified polyclonal anti-TGF-β2 Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Supernatants from BHK/HM-5, BHK/MKL (both gifts from Dr. J. Matous, University of Washington, Seattle, WA), and WEHI 3B (gift from Dr. S. Tsai, University of Utah, Salt Lake City, UT) cells were used as a source of GM-CSF, KL, and IL-3, respectively.

Isolation of hematopoietic progenitor and stem cells from bone marrow

Femurs and tibias were flushed with IMDM (Invitrogen Life Technologies, Grand Island, NY) supplemented with 5% FCS. Low density bone marrow cells, obtained after density centrifugation, were stained with Ter119, CD2, CD3, CD4, CD8, B220, Mac1, and Gr1. For 20 min at 4°C, washed, and stained with FITC-conjugated goat anti-rat Abs for 20 min at 4°C. After washing, the cells were stained for 20 min at 4°C with PE-conjugated Scal and biotin-conjugated CD117 (c-Kit). Washed with PBS, and stained with streptavidin-CyChrome. The cells were sorted on a MoFlo (DakoCytomation, Fort Collins, CO) or a FACSVantage SE (BD Biosciences, Mountain View, CA) flow cytometer at 30 PSI sheath pressure and at a rate of 12,000–15,000 events/s. Lin-Scal+ Kit+ (LSK) cells were sorted as cells with a low side scatter, a low to medium forward scatter (Fig. 1, R1), a green (lineage) fluorescence lower than the median fluorescence of cells stained with isotype-matched control Abs, an orange (Scal) fluorescence twice the intensity (in terms of channel numbers) of the brightest cells in control samples (Fig. 1, R2), and a positive red (c-Kit) fluorescence (Fig. 1, R3). Bone marrow pooled from at least two mice was used in each experiment. As the goat anti-rat secondary Ab was not blocked with rat immune globulins, most Lin− cells appear Scal positive in Fig. 1. This blocking step was omitted because only lineage-negative cells were isolated, and the blocking step did not affect the fluorescence or the number of cells in the sort windows in preliminary experiments.

Proteinase K treatment and dialysis of FCS

FCS was treated with 0.5 mg/ml proteinase K (Sigma-Aldrich, St. Louis, MO) for 30 min at 37°C, followed by heat activation at 65°C for 1 h. FCS was dialyzed using a MolecuPor membrane with a 5.5-kDa cutoff (Spectrum Laboratories, Rancho Dominguez, CA) against three changes of PBS over 48 h.

Culture of LSK cells

Sorted LSK cells, pooled from at least two mice in each experiment, were cultured in triplicate at 20–40 cells/well in flat-bottom, 96-well plates in IMDM supplemented with either 10% FCS (serum-containing medium (SCM)) or a serum replacement medium (StemPro (Invitrogen Life Technologies) serum-free medium (SFM)), 100 mg/ml penicillin/streptomycin, and 50 ng/ml each of Flt3L, TPO, and KL, unless mentioned otherwise. Three hours after plating, the exact number of cells per well was determined by visually counting the cells × 40 magnification. After 5 days of liquid culture at 37°C and 5% CO2, the cells were again counted, and in some experiments 500 cells were plated in methylcellulose cultures containing IMDM, IL-3 (10% WEHI 3B supernatant), GM-CSF (10% of BHK/HM-5 supernatant), KL (10% BHK/MKL supernatant), erythropoietin (2 U/ml), FCS (10%), anti-TGF-β (10 μg/ml), and α-thiglycerol (10−6 M). After 8-day incubation at 37°C in a humidified incubator with 5% CO2, the cultures were scored for colony formation. Most secondary colonies were myeloid, with 20–40% macroscopic colonies derived from high proliferative potential cells (CFC). Colony-forming cells (CFC) were purified with cellular proliferation in the liquid culture phase in every experiment (see Fig. 3a and Fig. 5, a and b), and determination of CFC output was therefore omitted in many experiments, including linkage analysis.

Statistical analysis

Student’s t test for paired samples was used unless indicated otherwise. All results are expressed as the mean ± SEM. A value of p < 0.05 was considered indicative of a statistically significant difference. For the comparison of multiple culture conditions, one-way ANOVA with Bonferroni correction for multiple testing was used.

Linkage analysis in BXD recombinant inbred strains

Linkage analysis was performed in BXD RI strains. These are commercially available and were generated by repeated inbreeding of F2 mice derived from the inbred progenitor strains, C57BL/6 and DBA/2. The resulting genome of RI strains is composed of a patchwork of homozygous chromosome segments derived from either progenitor strain, with each of the RI lines having a unique combination of patches from the progenitors (35). Each RI strain of a set is thus inbred and genetically distinct from the other strains in the set, because they inherited different combinations of chromosomal regions from each progenitor. Therefore, if a trait is determined by multiple genes, then the trait value will show a continuous distribution across a given set of RI strains. The distribution of the trait value among the RI strains is called the strain distribution pattern (SDP). Polymorphic markers derived from either one of the progenitors will also show a strain distribution pattern. Linkage analysis consists of determining which polymeric markers the phenotypic SDP shows a statistically significant correlation. This analysis is performed using webQTL (www.webqtl.org) (36). This web-based software for complex trait analysis uses an updated, error-checked database and is based on the Mapmanager software developed by Manly et al. (37). It statistically analyzes the linkage of a given trait with previously typed polymeric loci in the RI strains, which are inherited from either parental strain. The association between a marker and a trait is indicated by the likelihood ratio statistic (LRS) and an associated p value for point-wise linkage. However, the association of a phenotypic SDP with the SDP of hundreds of polyorphic markers is measured. Therefore, a close or identical match in SDPs may occur by chance (38, 39), and this probability is higher if more markers are tested. This would lead to a very high number of false positive QTls. To establish the genome-wide, as opposed to the point-wise, significance level of the association between a phenotypic and a marker SDP, a correction for multiple testing has to be introduced. One way to do this is to calculate the genome-wide probability of obtaining the observed linkage by random chance corresponding to a given error threshold using the nonparametric permutation method developed by Churchill and Doerge (38). In permutation testing, the phenotypic data are permuted 1000–5000 times, and the frequency of obtaining linkage anywhere in the genome at a given LRS value is calculated. This allows the determination of minimum LRS values to consider linkage suggestive (p < 0.5) or significant (p < 0.05) on a genome-wide basis. This algorithm is automatically implemented in the

![FIGURE 1. Isolation of LSK cells. Representative example of the sort windows used for the isolation of LSK cells.](http://www.jimmunol.org/.../figure1.jpg)
dose-response of TGF-
whether replicative stress induced in young mice would affect the
is subject to replicative senescence (43). We therefore investigated
cating that this cell population, which is highly enriched in HSC,
for the observed phenotype (40). Finally, webQTL also allows for com-
interval mapping, where linkage analysis is performed after removing
variation caused by other linked QTL in the genome.

Results
Stimulatory effect of TGF-β2 on LSK cells increases in a mouse
strain-dependent fashion with age and after replicative stress
We measured the responsiveness to TGF-β2 of LSK cells from aged
C57BL/6 and DBA/2 mice. Compared with 8-wk-old mice, baseline proliferation of LSK cells supported by the early acting cytokines, KL, Flt3L, and TPO (41, 42), was decreased ~3-fold in 18-mo-old C57BL/6 mice and 2-fold in DBA/2 mice (not shown) (21). As we have reported previously (11), the TGF-
18-mo-old C57BL/6 mice and 2-fold in DBA/2 mice (not shown)
cytokines, KL, Flt3L, and TPO (41, 42), was decreased
aged C57BL/6 and DBA/2 mice. Compared with 8-wk-old mice,
speciﬁc and mouse strain-dependent changes in the effect of
TGF-β2 on LSK cells as did aging. These data suggest that the responsiveness of LSK cells to the effect of TGF-β2 increases in a genetically determined fashion after replicative stress.

Because the TGF-β2 dose response showed clear genetically determined variation in aged C56BL/6 and DBA/2 mice, we next performed linkage analysis by testing the effect of TGF-β2 at 0.01 and 0.1 ng/ml on the proliferation in SCM of LSK cells from 23 BXD RI mouse strains (see Materials and Methods) at the age of 18 mo (Fig. 4). As both progenitors, C57BL/6 and DBA/2, express
the same allele of the Ly6A (Scal1) gene, comparing puriﬁed LSK
cells among BXD RI mice is justiﬁed (47). Our previously re-
ported linkage analysis of young BXD mice was performed at a
TGF-β2 concentration of 0.1 ng/ml (11). It can be seen in Fig. 4a
that at this concentration of TGF-β2 (which is inhibitory in young
mouse; see Fig. 2a), the relative inhibition of proliferation of LSK
cells is weaker in old than in young mice in most BXD RI strains.
The data obtained in old BXD mice at both 0.01 and 0.1 ng/ml
TGF-β2 in SCM are shown in Fig. 4b. The phenotypic variation in the effect of TGF-β2 on the proliferation in SCM of LSK cells from 18-mo-old BXD mice was more pronounced than that in the two progenitor strains, C57BL/6 and DBA/2 (solid lines in Fig. 4b). This typically occurs for multigenic traits, where the progen-
itor strains have a mixture of alleles with positive and negative contributions to the observed trait value. Some of the RI strains can

Aging of HSC is probably due to replicative senescence, be-
cause HSC have been shown to cycle slowly throughout life (29).
Furthermore, in the human system, progressive age-related telomere shortening in CD34+CD38− cells has been observed, indicat-
ing that this cell population, which is highly enriched in HSC, is subject to replicative senescence (43). We therefore investigated whether replicative stress induced in young mice would affect the dose-response of TGF-β2 on LSK cells. The cytotoxic drug 5-FU (150 mg/kg) was administered three times at 14-day intervals. Two
weeks after the last injection, LSK cells were isolated. 5-FU erad-
icates cycling cells, thereby inducing enhanced cycling activity in quiescent HSC to promote hematopoietic recovery (44, 45). A sim-
ilar treatment has been shown to decrease the reconstitution activ-
ity of HSC (46). As in LSK cells from aged mice (21), proliferation and CFC generation were 2- to 3-fold lower in LSK cells from mice treated with 5-FU than in cells from mice treated with PBS in both C57BL/6 and DBA/2 mice (Fig. 3a). The stimulatory effect of TGF-β2 on LSK cells increased in 5-FU-treated C57BL/6 mice, whereas in DBA/2 mice, the change in the TGF-β2 dose response was less pronounced (Fig. 3b). No changes were observed in the dose response of TGF-β1 in either mouse strain (not shown). The TGF-β2 dose response on the proliferation of LSK cells was similar in cells isolated 4–6 days after a single injection of 5-FU or PBS, indicating that acute hematopoietic stress does not affect the response of LSK cells to TGF-β2 (not shown). Repeated recruit-
ment of stem and progenitor cells thus induced similar isoform-
speciﬁc and mouse strain-dependent changes in the effect of
TGF-β2 on LSK cells as did aging. These data suggest that the responsiveness of LSK cells to the effect of TGF-β2 increases in a genetically determined fashion after replicative stress.

FIGURE 2. Effect of aging on the dose response of TGF-β2. Dose
responses of TGF-β2 on proliferation in 5-day cultures of LSK cells from 18-mo-old and 8-wk-old C57BL/6 (a) and DBA/2 (b) mice, supported by KL, Flt3L, and TPO (n = 4). *, Signiﬁcantly different from 8-wk-old mice.

FIGURE 3. Effect of repeated hemopoietic stress on the TGF-β2
dose response in SCM. a, Proliferation and CFC generation in LSK cells from C57BL/6 and DBA/2 mice after repeated treatment with 5-FU. Results are expressed as a percentage of the value in control mice treated with PBS in same experiment. b, Dose response of TGF-β2 on the proliferation sup-
ported by KL, Flt3L, and TPO of LSK cells from C57BL/6 and DBA/2
mice treated with repeated administration of 5-FU or PBS (n = 4, each experiment consisting of three mice treated with 5-FU and three control mice treated with PBS in both panels). †, Signiﬁcantly different from DBA/2.
FIGURE 4. Linkage analysis in aged BXD RI strains. a, Strain distribution pattern of the effect of TGF-β2 on KL-, Flt3L-, and TPO-supported proliferation of LSK cells (9252) from 8-wk-old (11) and 18-mo-old BXD mice (n = 1–4; LSK cells isolated from the pooled bone marrows of at least two mice were used in each individual experiment; no error bars are shown because in many cases, only one or two independent experiments were performed). b, Effect of TGF-β2 at 0.1 and 0.01 ng/ml on KL-, Flt3L-, and TPO-supported proliferation of LSK cells from 18-mo-old BXD mice (n = 1–3; LSK cells isolated from the pooled bone marrows of at least two mice were used in each individual experiment; no error bars are shown because in many cases, only one or two independent experiments were performed). c, Correlation between the age-related change in the effect of TGF-β2 at 0.1 ng/ml (a) and mean life span, as determined by Gelman et al. (48) and publicly available on webQTL (www.webqtl.org) (36).

then display more extreme phenotypes than the progenitor strains if they have accumulated most of the positive or most of the negative alleles for that trait (35). A QTL for the effect of TGF-β2 at 0.01 ng/ml was identified on chromosome 2 between 41 (D2Mit56) and 53 (D2Nds1) cM (maximum likelihood ratio statistic, 13.0). The genome-wide significance level of this QTL was in the suggestive range, as evaluated by permutation analysis (38, 39). There was no correlation between the effects of TGF-β2 in young and old mice (not shown). Accordingly, this locus does not coincide with the QTL, contributing to the effect of TGF-β2 in young mice we have previously identified (11). Quantitative trait analysis of the difference between the effect of TGF-β2 (0.1 ng/ml) in young and old mice (see Fig. 4a), although clearly subject to extensive genetically determined variation, did not yield any suggestive QTL. This is not unusual given the relatively limited number of BXD RI strains available and does not mean that there are no other QTL (35). However, we and others have previously shown that many quantitative traits affecting the hematopoietic stem cell compartment correlate with or map to similar chromosomal regions as life span (21, 32–34), a trait that shows clear quantitative genetic variation among BXD mice (48). For 11 BXD strains, publicly accessible life span data (36) compiled by Gelman et al. (48) and data on the effect of TGF-β2 on LSK cells of both young and old mice generated by us (see Fig. 4a) were available. Interestingly, a strong and statistically significant correlation was observed between the age-related increase in the effect of TGF-β2 on LSK cells and the mean life span (Fig. 4c). These data support the idea that traits affecting the hematopoietic stem cell compartment are closely linked to genetic variation in life span (21–23, 32–34) and suggest that TGF-β2 signaling in LSK cells may be mechanistically involved.

Isoform-specific effect of serum on the dose response of TGF-β in LSK cells is mediated by one or more low m.w., nonprotein factors

The dose responses of TGF-β1 and TGF-β3 on the proliferation of LSK cells, which are inhibitory, are strikingly different from the dose response of TGF-β2, which is biphasic, with a stimulatory effect at low concentrations of TGF-β2 (11). Although the proliferation of LSK cells in vitro is mainly differentiative, the positive regulatory effect of TGF-β2 on LSK cells in vitro appears relevant in vivo, because Tgfb2−/− mice have a defect in HSC function and cycling activity (11). Quantitative trait analysis furthermore suggested a role for TGF-β2 in the aging of stem and progenitor cells and possibly also in the determination of life span (see Fig. 4). To begin to elucidate the mechanistic basis for the differential responsiveness of LSK cells to TGF-β2 compared with TGF-β1 and TGF-β3, we tested the effects of TGF-β2 and TGF-β1 in liquid cultures containing 10% FCS (SCM), or a serum replacement (SFM). Cell number and CFC generation after 5 days of culture in the absence of TGF-β were similar in SCM and SFM (10,300 ± 1,300 cells and 4,125 ± 749 CFC/50 input LSK cells in SCM, and 8,167 ± 927 cells and 4,894 ± 700 CFC/50 input LSK cells in SFM, respectively; n = 30). In contrast to SCM, TGF-β2 at any concentration tested was a potent inhibitor of proliferation and CFC generation of LSK cells in SFM (Fig. 5, a and b). Furthermore, the presence of serum had only a limited effect on the dose response of TGF-β1, which was inhibitory at all concentrations in both SCM and SFM (Fig. 5, a and b). The small difference in the TGF-β1 dose response between SCM and SFM only reached statistical significance (p < 0.05) for the cellular proliferation of LSK cells at a TGF-β1 concentration of 0.001 ng/ml (Fig. 5a). We next tested the effect of neutralization of endogenously produced TGF-β in cultures of LSK cells in SFM. We have previously shown that TGF-β1 and TGF-β2 are expressed in LSK cells and that in SC, neutralizing anti-TGF-β2 Abs inhibited, whereas neutralizing anti-TGF-β1 Abs increased the proliferation of LSK cells, again emphasizing the qualitatively different effects of TGF-β1 and TGF-β2 on LSK cells (11). In SFM, however, both anti-TGF-β1 and anti-TGF-β2 Abs enhanced the proliferation and CFC generation of LSK cells (Fig. 5c). Taken together, these data indicate that one or more serum factors confer a stimulatory effect on the proliferation of LSK cells to both endogenous and exogenously added TGF-β2, but not, or only minimally so, to TGF-β1. The serum factors involved thus appear to modulate TGF-β signaling in an isoform-specific fashion.

To determine the nature of the serum factors that confer a stimulatory effect to TGF-β2, we tested the effect of addition of heat-treated and proteinase K-treated FCS to SFM (Fig. 5d). Treatment of FCS with proteinase K followed by heat treatment (95°C) did
that unknown serum factors dramatically enhance the proliferation of LSK cells in a dose-dependent fashion when TGF-β2 is present, but not, or much less so, in its absence.

The phenotype of Tgfb2−/− mice suggested that the serum-dependent effect of TGF-β2 is relevant in vivo (11). Therefore, we investigated whether mouse serum affects the TGF-β2 dose response in the same way as does FCS. As shown in Fig. 5f; addition of mouse serum to SFM caused a change in the TGF-β2 dose response, so that at low concentrations of TGF-β2, a stimulatory effect was observed. As with FCS, addition of mouse serum only minimally affected the TGF-β1 dose response (Fig. 5f). The effect of FCS on the TGF-β responsiveness of LSK cells could thus be reproduced with mouse serum. It is therefore likely that the factors present in FCS that regulate TGF-β2 signaling are also present in mouse serum and are not species specific in their effects.

Mouse strain-dependent variation in the TGF-β2 dose response is dependent on serum factors

We have shown previously that in SCM, LSK cells from BALB/c mice are significantly more responsive to the stimulatory effect of TGF-β2 than LSK cells from C57BL/6 and DBA/2 mice, whereas there is no strain-dependent variation in the effects of TGF-β1 on LSK cells (11). As the TGF-β2 dose response on LSK cells is affected by serum factors, we examined whether the mouse strain-dependent variation in the TGF-β2 dose response also occurred in SFM. Under these conditions, however, TGF-β2 potently inhibited the proliferation of LSK cells from all three mouse strains, and no significant genetically determined variation in the TGF-β2 dose response was observed (Fig. 6a). In contrast to TGF-β2, the dose response of TGF-β1 on the proliferation of LSK cells was inhibitory, was not affected by the presence of serum, and was not subject to mouse strain-dependent variation (Fig. 6b). The genetically determined variation in the TGF-β2 dose response is thus

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**FIGURE 6.** Absence of genetic and age-related variation in the effect of TGF-β2 in SFM. a and b, Dose responses of TGF-β2 (a) and TGF-β1 (b) on proliferation in 5-day cultures of LSK cells from the mouse strains listed at the top of the figure, supported by KL, Flt3L, and TPO in SFM and SCM (data in SCM are from Ref. 11), respectively (n = 3–13, except for BALB/c at 0.001 ng/ml both TGF-β1 and TGF-β2 in SFM, where n = 1). *, Significantly different from DBA/2 and C57BL/6 in SCM. All mice were 8–10 wk old. For all strains, every data point of the TGF-β2 dose response in SCM was significantly different from the corresponding data point in SFM, whereas for TGF-β1, no significant difference were observed between SCM and SFM, except for C57BL/6 at 0.001 ng/ml TGF-β1. c, Dose response of TGF-β2 in SFM, SFM supplemented with 10% FCS, or SFM supplemented with serum from 8-wk-old or 18-mo-old mice on LSK cells from 8-wk-old and 18-mo-old mice, respectively. See text for statistical analysis.

**FIGURE 5.** Role of serum factors in the effect of TGF-β2 on LSK cells in vitro. a and b, Dose responses of TGF-β1 and TGF-β2 on proliferation (a) and CFC generation (b) in 5-day cultures of LSK cells from 8-wk-old C57BL/6 mice, supported by KL, Flt3L, and TPO in SFM and SCM, respectively (mean ± SEM; n = 6–19). *, Significantly different from SFM. **a**, significantly higher than control without TGF-β2. c, Effects of affinity-purified polyclonal anti-TGF-β2 and monoclonal anti-TGF-β1 Abs on proliferation and CFC generation in cultures of LSK cells supported by KL, Flt3L, and TPO in SFM (mean ± SEM; n = 5). *, Significantly different from cultures with control Abs. d, Effect of addition of untreated FCS (SCM), FCS treated with proteinase K followed by heat-inactivation at 95°C (SCM prot. K + H1), or FCS after dialysis with a cutoff of 3.5 kDa (SCM dialys). e, Effect of addition of increasing concentrations of FCS to SFM on the proliferation of LSK cells supported by KL, Flt3L, and TPO in the presence and the absence of TGF-β2 at 0.1 ng/ml. The results of an experiment performed in triplicate, representative of three experiments, are shown. f, Dose response of TGF-β1 and TGF-β2 on proliferation in 5-day cultures of LSK cells from C57BL/6 mice supported by KL, Flt3L, and TPO in SFM or SFM supplemented with mouse serum (MS), respectively (mean ± SEM; n = 4). *, Significantly different from SFM.
dependent on factors present in serum, which act in an isoform-specific fashion.

Age-related change in TGF-β2 dose response is dependent on serum, and biological activity of the serum factor modulating the TGF-β2 dose response increases with age

We next tested whether the age-related change in the TGF-β2 dose response is also dependent on serum factors. In the absence of serum, the dose response of TGF-β2 was similar in old and young C57BL/6 mice (Fig. 6c) and was similar to that of TGF-β1 (not shown). The isoform-specific, age-related change in the TGF-β2 dose response and the genetically determined variation therein are thus also strictly dependent on the presence of serum factors. Similarly, the change in the TGF-β2 dose response after hemopoietic stress induced by repeated administration of 5-FU was dependent on the presence of serum factors, which act in an isoform-specific fashion because no change was observed in SFM, whereas the TGF-β1 dose response was not affected in either SCM or SFM (not shown).

As the responsiveness of LSK cells to the serum-dependent stimulatory effect of TGF-β2 increased with age, we investigated whether the activity in mouse serum that modulates the effect of TGF-β2 on LSK cells also increases with age. Addition of serum from 18-mo-old mice caused a larger shift in the TGF-β2 dose response than did addition of serum from 8-wk-old mice in cultures of LSK cells from both old and young mice (Fig. 6c). These data indicate that the biological activity of the factors in serum that confer a stimulatory effect to TGF-β2 on the proliferation of LSK cells increases with age.

Discussion

In this report we show that the positive regulatory effect of TGF-β2 on hemopoietic stem and progenitor cells increases with age in a mouse-strain dependent fashion correlating with longevity, and that the unique effect of TGF-β2 on LSK cells is entirely dependent on factors present in mouse and FCS. Taken together, our data suggest a role for TGF-β2 and for as yet unknown serum factors in the age-related changes in HSC and perhaps in longevity.

We have shown previously that the dose response of TGF-β2 on the proliferation and the CFC generation of LSK cells supported by early acting cytokines in the presence of serum is biphasic, with a stimulatory effect at low concentrations and a dose-dependent inhibitory effect at higher concentrations of TGF-β2 (11). A stimulatory effect of both TGF-β1 and TGF-β2 has been described on the proliferation and differentiation of committed granulocytic progenitors in response to GM-CSF (49, 50). These effects are probably different from the isoform-specific stimulatory effect of TGF-β2 we described in this study and in a previous report (11), because the targets for this positive regulatory effect of TGF-β2 are more primitive cells responsive to early acting cytokines and are capable of serial reconstitution in vivo. In this study we show that the stimulatory component of the TGF-β2 dose response is dependent on serum factors. Therefore, one explanation for the biphasic nature of the TGF-β2 dose response in SCM is that the serum-dependent stimulation of the proliferation of LSK cells, which is predominant at low concentrations of TGF-β2, is overridden at higher concentrations by the potent intrinsic inhibitory effect of TGF-β on the proliferation of LSK cells. The effect of serum on the dose response of TGF-β2 was, to a large extent, isoform specific. Tgfb2+/- mice have a slightly lower bone marrow cellularity and a more pronounced decrease in the frequency of LSK cells compared with wt littermates. Furthermore, Tgfb2+/- HSC cycle more slowly in vivo and have a defect in serial repopulating potential compared with wt HSC (11). These data strongly suggest that the serum-dependent stimulatory effect of TGF-β2 is relevant in vivo. This contention is further supported by the observation that the effect of mouse serum on the TGF-β2 dose response was similar to that of FCS. Another way to interpret the effect of serum is that serum factors potently enhance the proliferation of LSK cells in the presence of TGF-β2, but not, or less so, in its absence (see Fig. 5e). As TGF-β2 is expressed in the bone marrow and in purified LSK cells (11), these serum factors may, in fact, play a critical role in hemopoiesis in vivo. The observation that the response of LSK cells to TGF-β2 in the presence, but not in the absence, of serum shows mouse strain-dependent variation and undergoes genetically determined age-related changes suggests that the serum factors involved may act directly on LSK cells. It is therefore possible that it is the response to these as yet unidentified serum factors that is actually subject to mouse strain-dependent and age-related variation. Our data suggest that the factors involved have a low m.w. and are not proteins or peptides. A further characterization of these serum factors may be critical to our understanding of the biology of HSC.

As long as the identity and the signaling mechanisms of the unknown serum factors that regulate the effects of TGF-β2 on LSK cells are unknown, it will be difficult to precisely assess the role of the age-related increase in the responsiveness to TGF-β2 and serum factors in the aging of HSC in vivo. Furthermore, heterogeneity within the LSK compartment, such that LSK cells from DBA/2 mice may not be equivalent to LSK cells from C57BL/6 mice, may complicate interpretation of the observed genetic variation and age-related changes in TGF-β2 responsiveness. Phillips et al. (51) have indeed shown differential Rho123 staining of LSK cells from DBA/2 and C57BL/6 mice. It cannot be excluded that a subpopulation of LSK cells that responds to TGF-β2 with enhanced proliferation is relatively depleted in old DBA/2 mice and is enriched in old C57BL/6 mice. We hypothesize that TGF-β2 signaling is involved in the maintenance of HSC function with age. In very old C57BL/6 mice, the cycling activity of HSC in vivo is increased compared with that in young mice (24). The number of day 35 cobble stone area-forming cells (CAFCd35), a surrogate in vitro assay for HSC, increases in old C57BL/6 mice, but decreases in old DBA/2 mice compared with young mice (22, 23). Finally, it has been shown that competitive repopulation capacity of HSC is maintained (25) or increases (24) in aged C57BL/6 mice, but shows an age-related decline in DBA/2 mice (25). These findings were consistent with the observation that in embryo-aggregated D2->B6 mice, DBA/2-derived hemopoiesis is eclipsed upon aging by C57BL/6-derived hemopoiesis through stem cell-intrinsic mechanisms (3, 4, 51). Taken together, these data suggest that stem cell function is maintained better in long-lived C57BL/6 mice than in the shorter-lived DBA/2 mice. We have shown that TGF-β2 is important for maintenance of the serial reconstitution potential of HSC (11), a procedure that may induce replicative aging (31). Therefore, it is possible that the age-related increase in the responsiveness to the positive regulatory effect of TGF-β2 contributes to the maintenance of HSC function in aged C57BL/6 mice. It is interesting to note in this context that the BXD mouse strain with the highest number of CAFCd35 in old mice (BXD15) (22) is also the strain with the highest responsiveness to the serum-dependent stimulatory effect of TGF-β2 when old (see Fig. 4b). This trait mapped to chromosome 2 with a suggestive level of genome-wide significance. Although suggestive QTL require confirmation (38), it is striking how many other QTL related to the hemopoietic stem and progenitor cell compartments were identified by us and other investigators on chromosome 2. These traits include the age-related change in the number of CAFCd35 (22,
32), the mobilization of progenitor cells (52), the proliferative capacity of LSK cells in young (9) and old mice (21), and the number of LSK cells in young mice (9). All these QTL occur within a genetic distance of ~30 cm. Further research will be needed to clarify whether these are all separate QTL.

We and others have previously reported that quantitative variation in the hematopoietic stem cell compartment and in life span show close genetic linkage, suggesting a role for HSC, or possibly for somatic stem cells in general, in the regulation of life span (21–23, 32–34). It is possible that besides the responsiveness to the serum-dependent effect of TGF-β2 on LSK cells from young mice (11) and the age-related increase in the responsiveness of LSK cells to this effect, other aspects of the TGF-β2/serum cofactor system in LSK cells also show mouse strain-dependent variation. As the activity of the serum cofactor increases with age and as aging is mouse strain dependent (22, 32), it is possible that the activity of the serum cofactor is also subject to genetically determined variation. Furthermore, the expression or availability of TGF-β2 in the microenvironment may change with age in a genetically determined fashion. QTL, determining the intrinsic responsiveness of LSK cells to TGF-β2, may also be involved in the regulation of stem cell function in aged mice. Thus, our data raise the possibility that TGF-β2 may play a mechanistic role in determining longevity through its effects on HSC or on somatic stem cells in general.

References


