Transforming Growth Factor β Inhibits Endomitosis in the Dami Human Megakaryocytic Cell Line

By Sheryl M. Greenberg, Chittaranjan Chandrasekhar, David E. Golan, and Robert I. Handin

Megakaryocyte development is a carefully controlled process that is at least partially regulated by cytokines. Previous investigations of megakaryocyte development have focused primarily on defining growth factors that induce or enhance differentiation. In this study we demonstrate that a specific cytokine, transforming growth factor β1 (TGFβ1), inhibits the phorbol myristate acetate (PMA)-induced differentiation of the Dami human megakaryocytic cell line. The addition of purified platelet TGFβ1 inhibits PMA-induced endomitosis in a dose-dependent manner. Inhibition of endomitosis occurs with as little as 0.4 pmol/L TGFβ1, is half-maximal at 6.4 pmol/L, and is maximal between 40 and 200 pmol/L TGFβ1. Inhibition does not require other growth factors or nonmegakaryocytic cells. Removal of TGFβ1 from the cultures decreases inhibition, suggesting that the continuous presence of TGFβ1 is required and that its effects are reversible. This effect occurs even though the Dami cells constitutively express TGFβ1 messenger RNA (mRNA) and the TGFβ1 mRNA levels are increased by PMA. TGFβ1 also has been shown to inhibit endomitosis during short-term culture of primary human megakaryocytes. These results suggest a model in which negative as well as positive regulatory factors modulate a critical stage of megakaryocyte development.

MEGAKARYOCYTE development and platelet production is a carefully controlled complex process thought to be regulated at several stages.[1,2] Most investigations have focused on "positive" regulatory activities that can either stimulate megakaryocyte proliferation or induce terminal differentiation. Growth factors like interleukin-3 (IL-3) increase proliferation, ie, colony formation, of the earliest committed progenitors. This increases the pool of cells which develop into mature human megakaryocytes and platelets.[3,4] Growth factors such as IL-6, megakaryocyte stimulatory factor (MSF), thrombopoietic stimulatory factor (TSF), and megakaryocyte potentiator induce late murine and human megakaryocyte differentiation by enhancing platelet organelle and membrane development.[1,2,5-8] At the stage between colony formation and cytoplasmic development, megakaryocytes become polyploid by endomitosis, a process of repeated nuclear replication without concomitant cell division that is unique to the megakaryocytic lineage. There are previous reports that transforming growth factor β1 (TGFβ1) might also play a role in megakaryocytopoiesis. This is of particular interest because TGFβ1 is abundant in platelets and maturing megakaryocytes[9-13] and is released during platelet degranulation.[9,14-16]

The TGFβ family of polypeptides are multifunctional regulatory molecules that are synthesized by many different cells and for which nearly all cells have high-affinity receptors.[17,18] They have pleiotropic effects and inhibit proliferation of most cells, antagonize the mitogenic effects of some growth factors, and increase the expression of cell adhesion molecules and their receptors.[18-25] TGFβ1, the predominant form, is a homodimeric, 25-Kd polypeptide that inhibits murine and human hematopoietic progenitor cell proliferation in the presence of IL-3 or granulocyte-macrophage colony-stimulating factor in vitro[13,14,20,26] and in vivo.[19] TGFβ1 can also inhibit megakaryocyte colony formation.[14,26] In K562 cells, TGFβ1 inhibits erythroid proliferation and enhances erythroid differentiation as measured by hemoglobin synthesis.[27]

One approach to investigating the role of a growth factor like TGFβ1 in megakaryocytopoiesis would be to assess its effect on the differentiation of bone marrow megakaryocytes in short-term culture. However, such cultures contain other hematopoietic cells that could elaborate additional regulatory factors. To date, the only pure megakaryocyte assay system is one in which single megakaryocytes are cultured in individual microtiter culture wells.[26] However, with this system the limited number of cells precludes extensive analysis. Another approach would be to use megakaryocyte cell lines that respond to hematopoietic growth factors as a model of megakaryocyte development. We have previously established that the Dami cell line differentiates in response to phorbol myristate acetate (PMA) by becoming polyploid and increasing the expression of platelet membrane and granule constituents.[28] In this study we examine the effects of purified human platelet TGFβ1 on the differentiation of this cell line.

MATERIALS AND METHODS

Cell culture. Dami cells were cultured as previously described.[28] Briefly, cells were subcultured in Iscove's modified Dulbecco's medium (IMDM) containing 10% horse serum and 1% phytohemagglutinin-stimulated leukocyte-conditioned medium (PHA-LCM). PMA was used at a final concentration of 500 nmol/L. TGFβ1 was purchased from R & D Systems (Minneapolis, MN). Normal human bone marrow was obtained through Dr Joseph Antin (Bone Marrow Transplant Program, Brigham and Women's Hospital, Boston, MA), fractionated on Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ), and the nucleated cells were cultured overnight to remove adherent cells. The nonadherent cells were frozen for later use. The bone marrow cells were cultured in 10%

From the Hematology Division, Department of Medicine, Brigham and Women's Hospital, Boston; and the Departments of Medicine and Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA.

Submitted January 29, 1990; accepted April 12, 1990.

Supported in part by National Institutes of Health Grants P01-CA39542, P01-HL33014, R37-HL34787, and the William C. Moloney Fund, Brigham and Women's Hospital.

Address reprint requests to Sheryl M. Greenberg, PhD, Hematology Division, Brigham and Women's Hospital, 75 Francis St, Boston, MA 02115.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

0006-4971/90/7603-0001$3.00/0

horse serum, 1% PHA-LCM, in IMDM as described for the Dami cells.

Analysis of ploidy. Dami cells were seeded at 200,000 cells/mL and fed again after 2 days. Cells were harvested with trypsin 3 days later, and the nuclei were isolated, stained with propidium iodide, and analyzed on a Becton Dickinson FACS Analyzer (Mountain View, CA) as previously described.[28] Freshly prepared lymphocytes and propidium iodide standard beads (Flow Cytometry Standards Corp, Research Triangle Park, NC) were used to mark the position of the 2N cells. Cells were considered to be polyploid or undergoing endomitosis if the DNA content was greater than 4N.

Northern blot analysis. Total RNA was prepared by guanidine hydrochloride extraction and subjected to electrophoresis in 1% agarose formaldehyde denaturing gels as previously described.[28] Equal amounts of total RNA (10 μg) from each sample underwent electrophoresis and were transferred electrophoretically to GeneScreen (New England Nuclear, Boston, MA). A 28-base pair oligonucleotide which contains a sequence from the middle of exon 6 of the human TGFβ1 gene (R & D Systems), was labeled by kinase treatment of the 5′ end, and used for hybridization. The filters were washed for 20 minutes twice with 6X SSC (1X SSC = 150 mmol/L sodium chloride, 1.0% sodium dodecyl sulfate, and 15 mmol/L sodium citrate) and twice in 4X SSC at room temperature, and exposed to Kodak XAR film (Eastman Kodak, Rochester, NY) for autoradiography.

RESULTS

The Dami cell line normally contains primarily 2N and 4N cells. In the presence of 5% human platelet-poor plasma-derived serum, PMA treatment for 5 days induces 30% to 40% of the Dami cells to increase their DNA content to greater than 4N. The induced cells fall into discrete ploidy groups containing 8N, 16N, and 32N complements of DNA.[28] We found that the addition of between 1 and 5 ng/mL (40 and 200 pmol/L) TGFβ1 completely inhibited PMA-induced endomitosis (Fig 1). Concentrations of TGFβ1 ranging from 1 to 5 ng/mL (40 to 200 pmol/L) inhibited the PMA-induced endomitosis in a dose-dependent manner in the presence of the 5% plasma-derived serum. Half-maximal inhibition required 0.3 ng/mL (12 pmol/L) with the maximal inhibition at 1 to 5 ng/mL (40 to 200 pmol/L) (Fig 2). In the presence of human serum, TGFβ1 was a more potent inhibitor of endomitosis. This may be due to secretion of TGFβ1 by platelets during serum preparation. Thus, in the presence of human serum, half-maximal inhibition was

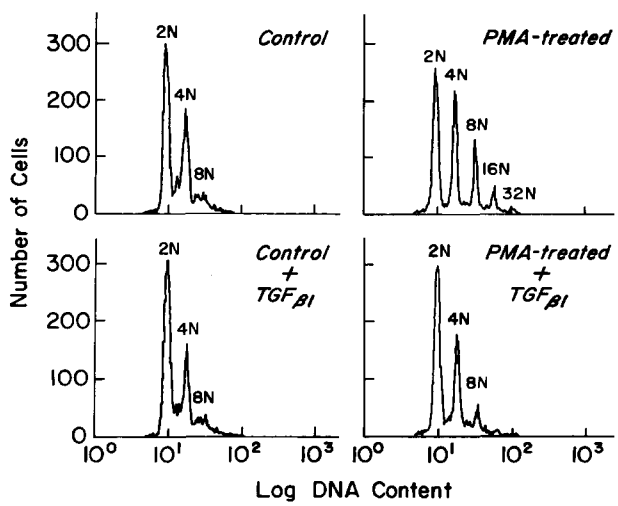

Fig 1. TGFβ1-mediated inhibition of PMA-induced endomitosis. Dami cells were cultured with 5% human plasma-derived serum for 5 days and the nuclei were stained with propidium iodide as described in Materials and Methods. Ten thousand cells were analyzed for each sample. The first major peak coincides with 2N cells such as lymphocytes. Subsequent peaks represent the proportion of cells with ploidies of 4N, 8N, 16N, 32N, and greater. Fluorescence intensity (*x*-axis) is proportional to DNA content.

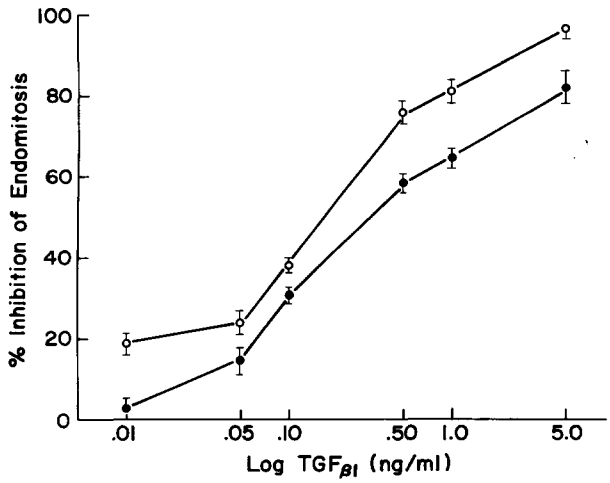

Fig 2. Percent inhibition by TGFβ1 of maximal PMA-induced endomitosis. Dami cells (seeded at 200,000/mL) were treated with PMA in the presence of 5% human platelet-poor plasma-derived serum (●) or human serum (○), and increasing concentrations of TGFβ1. The culture medium was replaced after 2 days, at which time PMA and TGFβ1 were replenished. After a total of 5 days, the nuclei were stained with propidium iodide and analyzed for ploidy distribution. Cells with amounts of DNA greater than that of a 4N cell were considered polyploid. Percent inhibition was calculated from the difference between maximal and TGFβ1-modulated PMA-induced endomitosis, and divided by the maximal PMA-induced endomitosis with serum or plasma in the absence of TGFβ1. The denominator was corrected for the proportion of cells greater than 4N in the unstimulated control cultures. This is a representative experiment.

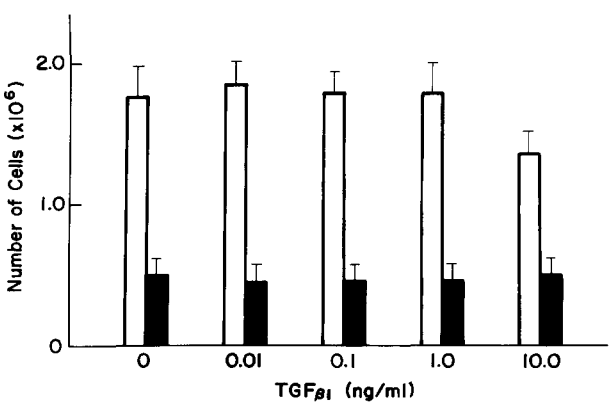

Fig 3. Dami cell proliferation in the presence of TGFβ1. After 5 days of culture in the presence of the indicated TGFβ1 concentrations as described in Fig 2, cells were harvested with trypsin and counted by hemacytometer. (□), Control cells (not stimulated with PMA); (■), PMA-differentiated cells.

Table 1. Effect of TGFβ1 on Endomitosis in Normal Human Megakaryocytes

Treatment	% Cells > 4N N = 4	(±SD)
None	2.50	(±0.47)
10 ng/mL TGFβ1	1.55	(±0.45)

achieved at 0.16 ng/mL (6.4 pmol/L) TGFβ1 (Fig 2). As shown in Fig 3, concentrations of TGFβ1 that inhibited endomitosis had no effect on the proliferation of control or PMA-treated Dami cells. A slight (18%) decrease in Dami cell proliferation at the higher TGFβ1 concentrations was noted in the control cells. When cell viability was assessed by trypan blue exclusion, the percentage of dead cells did not change over the entire range of TGFβ1 concentrations tested (data not shown).

To determine the physiologic relevance of the TGFβ1-mediated inhibition of endomitosis, the effect of TGFβ1 on normal human megakaryocytes in crude preparations of bone marrow cells was also analyzed. Normal human bone marrow was prepared by fractionation on Ficoll-Paque and cultured overnight to remove adherent cells. Preliminary experiments demonstrated that the only bone marrow cells which were capable of achieving a greater than 4N ploidy state were megakaryocytic by their positive reaction with anti-platelet GPIb and GPIIb/IIIa monoclonal antibodies (MoAbs). The addition of 10 ng/mL TGFβ1 (400 pmol/L) decreased the proportion of nucleated cells that spontaneously achieved a ploidy state greater than 4N during 5 days of culture from 2.50% to 1.55% (Table 1), a 37% inhibition of baseline endomitosis in normal human megakaryocytes.

To determine whether the effects of TGFβ1 on endomitosis were reversible, Dami cells were cultured in the presence of PMA and TGFβ1 for 2 days followed by PMA alone for 3 days. The cells treated in this way became polyploid to the same extent as cells treated with PMA alone for 3 days (Table 2), suggesting that the mechanism by which TGFβ inhibits endomitosis is reversible. In cultures induced with PMA for 5 days, TGFβ1 treatment for the first 2 days resulted in a higher ploidy distribution than TGFβ1 treatment for the entire 5 days (Table 2). This suggests that the continuous presence of TGFβ1 is required for maximal inhibition. Decreasing the concentration of cells had no effect on the extent of PMA-induced endomitosis or on the TGFβ1 inhibition (data not shown).

Table 2. Reversibility of TGFβ1-Mediated Inhibition Endomitosis

Treatment During Days 0-2	Treatment During Days 3-5	% Cells > 4N (±SD) N = 5	% Maximal Endomitosis* (±SD) N = 5
None	None	8.1 (±1.7)	0
None	PMA	20.0 (±1.8)	43.8 (±6.6)
PMA	PMA	35.4 (±1.4)	100.0
PMA + TGFβ1	PMA	19.8 (±1.2)	42.8 (±8.0)
PMA + TGFβ1	PMA = TGFβ1	13.3 (±2.1)	19.0 (±7.6)

TGFβ1 was used at a final concentration of 5 ng/mL; PMA was used at a final concentration of 500 nmol/L.

*Percent maximal endomitosis was calculated as the percent of cells that achieved ploidy levels greater than 4N in the presence of TGFβ1 divided by the maximal percent of cells greater than 4N achievable in the absence of TGFβ1.

To determine whether the Dami cells make TGFβ1 as has been demonstrated for normal megakaryocytes, total RNA was electrophoresed and transferred to filters for hybridization with a 28-nucleotide oligomer complementary to exon 6 of the human TGFβ1 gene. As shown in Fig 4, control and PMA-induced cells express the 2.2-kilobase TGFβ1 messenger RNA continuously with a slight increase in response to PMA. Incubation with an MoAb directed against TGFβ1 did not enhance the extent of endomitosis (data not shown).

DISCUSSION

Early megakaryocyte development is regulated by agents such as IL-3 that increase the proliferation of committed progenitor cells. As in other hematopoietic lineages, the proliferation of megakaryocyte progenitors in clonal assays is inhibited by TGFβ1.[14,26] Late megakaryocyte differentiation, including expression of platelet cytoplasmic membranes, granules, and stored components in polyploid megakaryocytes, is enhanced or induced by less well-defined growth factors like MSF, TSF, or IL-6.[1,2] TGFβ1 also inhibits this late stage of megakaryocyte maturation.[26,29] Positive regulatory factors that act on megakaryocytes at an intermediate stage of differentiation have not been clearly defined. Based on data derived from primary mixed-cell rat bone marrow cultures, it has been suggested that there may be an inhibitor of endomitosis; although to date no direct and clear regulation of human megakaryocyte endomitosis has been demonstrated in a purified cell system.[29,30] In this report, we have demonstrated that purified human TGFβ1 inhibits develop-

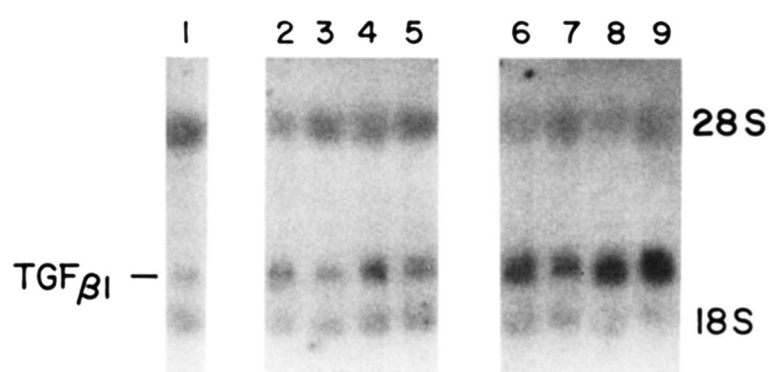

Fig 4. Northern blot analysis of Dami cell RNA. Ten micrograms of total RNA prepared from control and PMA-induced Dami cells at the indicated time points after the initiation of culture and PMA addition was subjected to electrophoresis in 1% agarose formaldehyde gels and electroblotted onto GeneScreen filters (New England Nuclear). The filters were hybridized with the radiolabeled oligonucleotide sequence complementary to human TGFβ1, washed, and autoradiographed. Lane 1, day 0 (before subculture); lanes 2 through 5, 1, 2, 3, and 4 days after subculture; lanes 6 through 9, 1, 2, 3, and 4 days after subculture in the presence of PMA.

ment of the polyploid state of the human megakaryocytic Dami cell line and that this inhibitory effect does not require the presence of other hematopoietic cells. TGFβ1 similarly inhibited megakaryocyte endomitosis in primary cultures of normal human bone marrow that contained cells of other hematopoietic lineages.

Relatively high levels of TGFβ1 have been found in areas of active tissue differentiation that contain hematopoietic stem cells, such as bone marrow and fetal liver,[31] and specifically in megakaryocytes.[31] As has been demonstrated in other cell lines with megakaryocytic properties,[27,32] TGFβ1 is expressed by the Dami cells. The level of expression is increased by PMA, an event that may be explained by the presence of three phorbol ester responsive elements (TREs) on the TGFβ1 gene.[33] Incubation of Dami cells with a monoclonal TGFβ1 antibody at levels sufficient to neutralize 50 ng TGFβ1 did not enhance the extent of endomitosis, suggesting that it may not be released extracellularly, but this does not exclude the possibility of autocrine regulation.

It is well-known that TGFβ1 inhibits DNA synthesis and proliferation of B cells, epithelial cells, and early bone marrow progenitors.[18,19] However, TGFβ1 did not affect Dami cell proliferation over the range of concentrations used to inhibit endomitosis. Thus, it appears that the mechanism of inhibition of endomitosis does not involve inhibition of DNA synthesis.

Of the four types of receptors for TGFβ that have been identified, two are well-defined. The high affinity TGFβ type I receptor is present on hematopoietic cells. It preferentially binds TGFβ1 and may be the principal cell surface molecule through which TGFβ1 induces its pleiotropic effects on signal transduction, altered gene expression, and changes in adhesion and growth control of hematopoietic cells.[13] The type II receptor is also a high affinity TGFβ1 receptor, and may also play a role in hematopoiesis.[13] It is possible that each receptor plays a specific role in mediating TGFβ1 action. Keller et al[22] have suggested that if one of these were the principal receptor through which TGFβ1 mediates its effects on proliferation, loss of expression of this receptor might account for the loss of negative growth regulation in some leukemias. However, since we have shown that differentiation of the Dami cells, like that of normal megakaryocytes, is inhibited by TGFβ1, it is possible that a different TGFβ receptor may be involved in regulating megakaryocyte differentiation.

In summary, we have described how the human megakaryocytic Dami cell line can be used as a tool to examine the regulation of megakaryocyte differentiation. Specifically, we have demonstrated that purified, exogenous TGFβ1 directly inhibits endomitosis but not proliferation in a pure megakaryocyte cell system. Because platelets contain abundant amounts of TGFβ1, this data supports the concept that megakaryocyte endomitosis may be regulated by a negative autocrine feedback mechanism.

ACKNOWLEDGMENT

The authors thank Dr David J. Kuter for helpful discussions. We are also very grateful to Amy Maurer for excellent technical assistance.

REFERENCES

1. Hoffman R: Regulation of megakaryocytopoiesis. Blood 74: 1196, 1989

2. McDonald TP: The regulation of megakaryocyte and platelet production. Int J Cell Cloning 7:139, 1989

3. Teramura M, Katahira J, Hoshino S, Motoji T, Oshimi K, Mizoguchi H: Clonal growth of human megakaryocyte progenitors in serum-free cultures: Effect of recombinant human interleukin 3. Exp Hematol 16:843, 1988

4. Bruno E, Briddell, Hoffman R: Effect of recombinant and purified hematopoietic growth factors on human megakaryocyte colony formation. Exp Hematol 16:371, 1988

5. Ishibashi T, Kimura H, Shikama Y, Uchida T, Kariyone S, Hirano T, Kishimoto T, Takatsuki F, Akiyama Y: Interleukin-6 is a potent thrombopoietic factor in vivo in mice. Blood 74:1241, 1989

6. Greenberg SM, Kuter DJ, Rosenberg RD: In vitro stimulation of megakaryocyte maturation by megakaryocyte stimulatory factor. J Biol Chem 262:3269, 1987

7. McDonald TP: Regulation of megakaryocytopoiesis by thrombopoietin. Ann NY Acad Sci 509:1, 1988

8. Williams N, Eger RR, Jackson HM, Neslson DJ: Two factor requirement for murine megakaryocyte colony formation. J Cell Physiol 110:101, 1982

9. Assioan RK, Komoriya A, Meyers CA, Miller DA, Sporn MB: Transforming growth factor-β in human platelets: Identification of a major storage site, purification, and characterization. J Biol Chem 258:7155, 1983

10. Alitalo R, Makela TP, Koskinen LC, Andersson LC, Alitalo K: Enhanced expression of transforming growth factor β during megakaryoblastic differentiation of K562 leukemia cells. Blood 71:899, 1988

11. Witte DP, Stambrook PJ, Feliciano E, Jones CLA, Lieberman MA: Growth factor production by a human megakaryocytic tumor cell line. J Cell Physiol 137:86, 1988

12. Lehnert SA, Akhurst RJ: Embryonic expression pattern of TGF beta type-1 RNA suggests both paracrine and autocrine mechanisms of action. Development 104:263, 1988

13. Ohta M, Greenberger JS, Anklesaria P, Bassols A, Massague J: Two forms of transforming growth factor beta distinguished by multipotential hemopoietic progenitor cells. Nature 329:539, 1987

14. Mitjavila MT, Vinci G, Villeval JL, Kieffer N, Henri A, Testa U, Breton-Gorius J, Vainchencker W: Human platelet alpha granules contain a nonspecific inhibitor of megakaryocyte colony formation: Its relationship to type β transforming growth factor (TGFβ). J Cell Physiol 134:93, 1988

15. Assoian RK, Sporn MB: Type β transforming growth factor in human platelets: Release during platelet degranulation and action on vascular smooth muscle cells. J Cell Biol 102:1217, 1986

16. Childs CB, Proper JA, Tucker RF, Moses HL: Serum contains a platelet-derived transforming growth factor. Proc Natl Acad Sci USA 79:5312, 1982

17. Sporn MB, Roberts AB, Wakefield LM, Assoian RK: Transforming growth factor-β: Biological function and chemical structure. Science 233:532, 1986

18. Massague J: The TGFβ family of growth and differentiation factors. Cell 49:437, 1987

19. Goey H, Keller JR, Back T, Longo DL, Ruscetti FW,

Wiltrout RH: Inhibition of early murine hemopoietic progenitor cell proliferation after in vivo locoregional administration of transforming growth factor-β1. J Immunol 143:877, 1989

20. Sing GK, Keller JR, Ellingsworth LR, Ruscetti FW: Transforming growth factor β selectively inhibits normal and leukemic human bone marrow cell growth in vitro. Blood 72:1504, 1988

21. Ignotz RA, Massague J: Cell adhesion protein receptors as targets for transforming growth factor-β action. Cell 51:189, 1987

22. Keller JR, Sing GW, Ellingsworth LR, Ruscetti FW: Transforming growth factor β: Possible roles in the regulation of normal and leukemic hematopoietic cell growth. J Cell Biochem 39:175, 1989

23. Heino J, Ignotz RA, Hemler ME, Crouse C, Massague J: Regulation of cell adhesion receptors by transforming growth factor-β: Concomitant regulation of integrins that share a common β1 subunit. J Biol Chem 164:380, 1989

24. Roberts CJ, Birkenmeier TM, McQuillan JJ, Akiyama SK, Yamada SS, Chen W-T, Yamada KM, McDonald JA: Transforming growth factor β stimulates the expression of fibronectin and of both subunits of the human fibronectin receptor by cultured human lung fibroblasts. J Biol Chem 263:4586, 1988

25. Ignotz RA, Endo T, Massague J: Regulation of fibronectin and type I collagen mRNA levels by transforming growth factor-β. J Biol Chem 262:6443, 1987

26. Ishibashi T, Miller SL, Burstein SA: Type β transforming growth factor is a potent inhibitor of murine megakaryocytopoiesis in vivo. Blood 69:1737, 1987

27. Chen LL, Dean A, Jenkinson T, Mendelsohn J: Effect of transforming growth factor-β1 on proliferation and induction of hemoglobin accumulation in K562 cells. Blood 74:2368, 1989

28. Greenberg SM, Rosenthal DS, Greeley TA, Tantravahi R, Handin RI: Characterization of a new megakaryocytic cell line: The Dami cell. Blood 72:1968, 1988

29. Kuter DJ, Gminski D, Rosenberg RD: Platelets contain several inhibitors of megakaryocyte growth and ploidization, in Breton-Gorius J (ed): Molecular Biology and Differentiation of Megakaryocytes. New York, NY, Liss, 1990 (in press)

30. Kuter DJ, Greenberg SM, Rosenberg RD: The analysis of megakaryocyte ploidy in rat bone marrow cultures. Blood 74:1952, 1989

31. Ellingsworth LR, Brennan JE, Folk K, Rosen DM, Bentz H, Piez KA, Seyedin SM: Antibodies to the N-terminal portion of cartilage-inducing factor A and transforming growth factor β. J Biol Chem 261:12362, 1986

32. Witte DP, Stambrook PJ, Feliciano E, Jones CLA, Lieberman MA: Growth factor production by a human megakaryocytic tumor cell line. J Cell Physiol 137:86, 1988

33. Scotto L, Vaduva PI, Wager RE, Assoian RK: Type β1 transforming growth factor gene expression. J Biol Chem 265:2203, 1990