Down-modulation of c-*kit* mRNA and protein expression by erythroid differentiation factor/activin A

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Abstract We examined the effects of erythroid differentiation factor (EDF)/activin A on the expression of c-*kit* mRNA and protein in murine erythroleukemia (MEL) cells. EDF/activin A induced MEL cells to benzidine positive cells. Northern blot analysis showed that the c-*kit* mRNA expression was reduced synchronously with increase of β-globin and uroporphyrinogen decarboxylase gene expression during EDF/activin A induced erythroid differentiation. Scatchard analysis indicated that the cell surface receptor number was reduced without change of affinity during differentiation. Our results suggest that EDF/activin A may act as a natural regulator of erythropoiesis with modulation of c-*kit* gene expression.

Key words: c-*kit*; Erythroleukemia; EDF/activin A; Differentiation

1. Introduction

All blood cells are derived from a few common pluripotent stem cells. Several cytokines regulate the proliferation and differentiation of hematopoietic stem cells. Erythroid differentiation factor (EDF), which was isolated from conditioned media of phorbol ester-treated THP-1 cells, has a potent differentiation-inducing activity for murine erythroleukemia (MEL) cells [1]. A subsequent study showed that EDF was identical to activin A that was first purified from ovarian fluid as a factor to stimulate the secretion of pituitary follicular-stimulating hormone [2]. Several reports have shown that activin A can modulate the colony formation of erythroid burst-forming units and erythroid colony-forming units [3–5]. In addition, Yamashita et al. have reported that bone marrow stromal cells produce EDF/activin A [6]. These results suggest that EDF/activin A possibly acts as a natural regulator of erythropoiesis in the bone marrow. However, the mechanism of the action of EDF/activin A on the process of erythropoiesis remains largely unclear.

The proto-oncogene c-*kit* [7,8], which is a receptor for stem cell factor (SCF) [9–14], plays a critical role in early hematopoiesis. Mutations at the murine dominant-white spotting locus that maps to c-*kit* cause severe anemia [15]. In addition, c-*kit* antisense oligonucleotides inhibit the colony-forming ability of CD34 positive cells cultured in the presence of interleukin-3 (IL-3) [16]. These results suggest that the regulators of c-*kit* expression can have profound effects on hematopoiesis. In this report, we demonstrated modulation of c-*kit* expression during EDF/activin A induced erythroid differentiation of MEL cells.

2. Materials and methods

2.1. Growth factors and probes

Recombinant EDF/activin A was kindly provided by Ajinomoto Co. Inc. Recombinant murine SCF was kindly provided by Kirin-Amgen Inc. SCF was iodinated by the chloramine-T method with a minor modification as described elsewhere [17]. Briefly, SCF (5 μg), 0.5 mCi of $Na^{125}I$, and chloramine-T (3.75 μg/ml) in 30 μl of 0.3 M sodium phosphate (pH 7.4) were placed in a 1.5-ml polypropylene tube. The iodination reaction was continued for 10 min at 22°C and stopped by addition of 5 μl of sodium metabisulfite (60 μg/ml). Labeled SCF was separated from free ^{125}I by column chromatography using a Sephadex G-25 (Pharmacia Fine Chemicals, Uppsala, Sweden). Murine c-*kit* cDNA was derived from mRNA of F5–5 murine erythroleukemia cells by reverse transcriptase polymerase chain reaction (RT-PCR) technique [18] with sense primer (5′-AGACGCTGACTATCAGCTC-3′) and antisense primer (5′-TGGCATCAGAGTTGGACA-3′) according to the previously published cDNA sequence [8]. Amplified 381 bp fragment was cloned into TA cloning vector (Invitrogen Co., San Diego, CA) and identified by DNA sequencing. Murine c-*kit*, β-globin [19], β-actin [20], uroporphyrinogen decarboxylase (UPD) [21] cDNA fragments were electrophoretically purified from agarose gels and labeled by using hexadeoxynucleotide random primers (Amersham International, Buckinghamsire, UK) with [α-32P]dCTP (specific activity 111 TBq/mmol; NEN Research Products, Boston, MA).

2.2. Culture and differentiation induction of MEL cells by EDF/activin A

A MEL cell line F5–5 [22] was maintained in Ham's F-12 medium containing 10% fetal calf serum (GIBCO, Grand Island, NY), penicillin (100 units/ml), and streptomycin (100 μg/ml) at 37°C in a humidified atmosphere containing 95% air, 5% CO_2. EDF/activin A (100 ng/ml) was added to the cell suspension (1×10^3/ml) at day 0 for differentiation induction [23]. On day 5, the cells were harvested for [^{125}I]SCF binding. The degree of differentiation was measured by scoring the ratio of benzidine staining positive cells.

2.3. Assay for binding of [^{125}I]SCF

F5–5 cells were incubated with [^{125}I]SCF in α-medium containing 0.1% bovine serum albumin, 20 mM HEPES, and 0.02% sodium azide (pH 7.4) at 15°C for 60 min. The cells were separated from unbound [^{125}I]SCF by centrifugation on a cushion of di-*n*-butyl phthalate. The radioactivity associated with the pellets was counted in an automatic gamma counter. Specific binding was determined by subtracting the non-specific binding measured in the presence of 100-fold excess unlabeled murine SCF from total binding.

2.4. Northern blot analysis

Total cellular RNA was prepared from F5–5 cells by the acid guanidium-thiocyanate phenol-chloroform method [24] and electrophoresed in 1% agarose gel containing formaldehyde. RNA was transferred to a nylon filter and hybridized with ^{32}P-labeled murine c-*kit*, UPD, β-globin, and β-actin cDNA as probes in 50% formamide, 3× SSC (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.4), 50 mM

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Tris-HCl (pH 7.5), 0.1% SDS, 20 μg/ml tRNA, 20 μg/ml boiled salmon sperm DNA, 1 mM EDTA, 0.02% bovine serum albumin, 0.2% polyvinylpyrolidone, and 0.02% Ficoll for 40 h at 37°C. The nylon membranes were washed with 2 × SSC, 1% SDS, and 1 × Denhardt at 37°C for 1 h, followed by 0.1 × SSC and 1% SDS at 50°C for 1 h and then underwent autoradiography using intensifying screens at −80°C.

3. Results

Fig. 1 shows the change in the binding of [^{125}I]SCF to F5–5 cells and ratio of benzidine positive cells during the erythroid differentiation induced by EDF/activin A. After treatment with 100 ng/ml of EDF/activin A, specific binding of SCF to F5–5 cells gradually decreased up to day 5. In contrast, differentiated erythroid cells producing hemoglobin appeared after day 2 and progressively increased in ratio. To clarify the observation obtained above, we tried saturation analysis of [^{125}I]SCF binding to both untreated and EDF/activin A-treated F5–5 cells. Scatchard analysis [25] of the binding data indicated a single class of binding sites with a dissociation constant (K_d) of 3.8×10^{-10} M and 2.8×10^{-10} M, respectively. The total number of binding sites were estimated at 2442/cell and 265/cell, respectively (Fig. 2). These results suggested that the apparent decrease in specific [^{125}I]SCF binding by EDF/activin A treatment was caused for a major part by a change in the binding sites in F5–5 cells.

Total RNA prepared from F5–5 cells after incubation with 100 ng/ml of EDF/activin A for up to 120 h, was hybridized with the murine c-*kit*, UPD, β-globin, and β-actin cDNA as probes (Fig. 3). Expression of β-globin and UPD mRNA significantly increased during erythroid differentiation. In contrast, expression of c-*kit* mRNA rapidly decreased during erythroid differentiation.

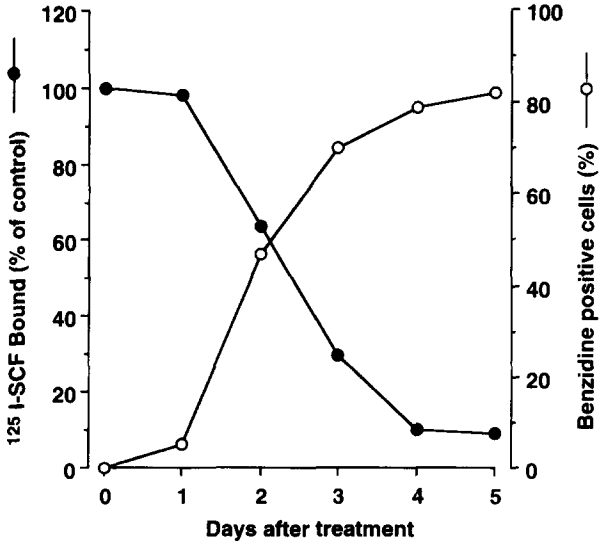

Fig. 1. Change in specific [^{125}I]SCF binding to F5–5 cells and ratio of benzidine-positive cells during EDF/activin A-induced erythroid differentiation. F5–5 cells were incubated with 100 ng/ml EDF/activin A and harvested after the indicated period. A portion of the cells was stained with benzidine dye, and the ratio of positive cells (●) was scored as the percentage of the total. Specific [^{125}I]SCF binding (○) was determined as described in section 2.

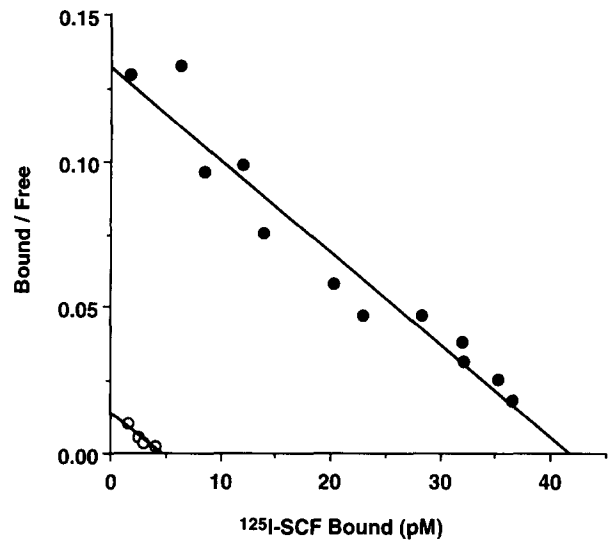

Fig. 2. Scatchard analysis of [^{125}I]SCF binding to F5–5 cells. The undifferentiated (●) and differentiated (○) cells (1×10^6) were incubated with increased concentrations of [^{125}I]SCF in the presence or absence of excess unlabeled SCF at 15°C for 60 min. Specific binding was determined as described in section 2. Each data point is the mean of duplicate determinations.

4. Discussion

In this report, we demonstrated that a potent erythroid differentiation inducer EDF/activin A down-regulated c-*kit* expression both at mRNA and protein levels synchronously with increase of β-globin and UPD during erythroid differentiation. SCF, the ligand for c-*kit*, enhances the growth of primitive hematopoietic progenitor cells synergistically with hematopoietic growth factors such as IL-3, granulocyte-macrophage colony-stimulating factor (GM-CSF), and granulocyte colony-stimulating factor in vitro [9–11]. These results suggest that SCF and c-*kit* system play an important role in early hematopoiesis. Anderson et al. have shown two forms of SCF that arise through differential RNA processing: a membrane-bound form and a soluble secreted form [11]. The transfected COS cells expressing murine surface SCF adhered with murine mast cells, suggesting that the membrane-bound molecule may act as an adhesion molecule [26]. Avraham et al. have reported that adhesion of human megakaryocytes could be mediated in part via their c-*kit* receptor binding to membrane-associated SCF expressed by bone marrow fibroblasts [27]. They have also reported that this interaction results in bone marrow fibroblast-stimulated megakaryocytic cell proliferation [27]. These reports suggest that early hematopoietic progenitor cells may be anchored to stromal elements within the bone marrow microenvironment suitable for the expansion by direct interaction of SCF to c-*kit*. Ogawa et al. have shown that the intensity of c-*kit* expression on bone marrow progenitor cells decrease with maturation [28]. Welham et al. have reported that IL-3, GM-CSF, and erythropoietin (EPO) down-regulate levels of c-*kit* mRNA [29]. Down-regulation of c-*kit* expression could be one of the molecular mechanisms mediated by cytokines, which causes the release of stem cells from the bone marrow into circulation when required, such as in hemorrhage or infection. Our findings, down-regulation of c-*kit* expression by EDF/activin A,

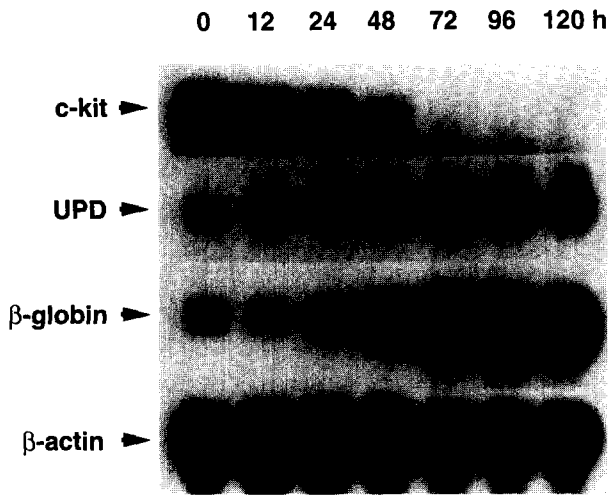

Fig. 3. Relative levels of c-*kit* mRNA in F5–5 cells during EDF/activin A-induced erythroid differentiation. F5–5 cells were incubated with 100 ng/ml EDF and harvested after the indicated period. Total RNA (20 μg) prepared from these cells were electrophoresed, transferred, and hybridized with murine c-*kit*, UPD, β-globin, and β-actin cDNA as the probes.

suggest that EDF/activin A may also play an important role in release of erythroid progenitor cells from bone marrow into circulation like EPO, when required. These possibilities should be addressed with further investigations.

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References

[1] Eto, Y., Tsuji, T., Takezawa, M., Takano, S., Yokogawa, Y. and Shibai, H. (1987) Biochem. Biophys. Res. Commun. 142, 1095–103.
[2] Vale, W., Rivier, J., Vaughan, J., McClintock, R., Corrigan, A., Woo, W., Karr, D. and Spiess, J. (1986) Nature 321, 776–779.
[3] Yu, J., Shao, L.E., Lemas, V., Yu, A.L., Vaughan, J., Rivier, J. and Vale, W. (1987) Nature 330, 765–767.
[4] Yu, J., Shao, L., Vaughan, J., Vale, W. and Yu, A.L. (1989) Blood 73, 952–960.
[5] Broxmeyer, H.E., Lu, L., Cooper, S., Schwall, R.H., Mason, A.J. and Nikolics, K. (1988) Proc. Natl. Acad. Sci. USA 85, 9052–9056.
[6] Yamashita, T., Takahashi, S. and Ogata, E. (1992) Blood 79, 304–307.
[7] Yarden, Y., Kuang, W.J., Yang, F.T., Coussens, L., Munemitsu, S., Dull, T.J., Chen, E., Schlessinger, J., Francke, U. and Ullrich, A. (1987) EMBO J. 6, 3341–3351.
[8] Qiu, F.H., Ray, P., Brown, K., Barker, P.E., Jhanwar, S., Ruddle, F.H. and Besmer, P. (1988) EMBO J. 7, 1003–1111.
[9] Zsebo, K.M., Williams, D.A., Geissler, E.N., Broudy, V.C., Martin, F.H., Atkins, H.L., Hsu, R.Y., Birkett, N.C., Okino, K.H., Murdock, D.C., Jacobsen, F.W., Langley, K.E., Smith, K.A., Takeishi, T., Cattanach, B.M., Galli, S.J. and Suggs, S.V. (1990) Cell 63, 213–224.
[10] Huang, E., Nocka, K., Beier, D.R., Chu, T.Y., Buck, J., Lahm, H.W., Wellner, D., Leder, P. and Besmer, P. (1990) Cell 63, 225–233.
[11] Anderson, D.M., Lyman, S.D., Baird, A., Wignall, J.M., Eisenman, J., Rauch, C., March, C.J., Boswell, H.S., Gimpel, S.D., Cosman, D. and Williams, D.E. (1990) Cell 63, 235–243.
[12] Martin, F., Suggs, S., Langley, K., Lu, H., Ting, J., Okino, K., Morris, C., McNiece, I., Jacobsen, F., Mendiaz, E., Birkett, N., Smith, K., Johnson, M., Parker, V., Flores, J., Patel, A., Fisher, E., Erjavec, H., Herrera, C., Wypych, J., Sachdev, R., Pope, J., Leslie, I., Wen, D., Lin, C.-H., Cupples, R. and Zsebo, K. (1990) Cell 63, 203–211.
[13] Flanagan, J.G. and Leder, P. (1990) Cell 63, 185–194.
[14] Williams, D.E., Eisenman, J., Baird, A., Rauch, C., Van, N.K., March, C.J., Park, L.S., Martin, U., Mochizuki, D.Y., Boswell, H.S., Burgess, G.S., Cosman, D. and Lyman, S.D. (1990) Cell 63, 167–174.
[15] Russel, E.S. (1979) Adv. Genet. 20, 357–459.
[16] Catlett, J.P., Leftwich, J.A., Westin, E.H., Grant, S. and Huff, T.F. (1991) Blood 78, 3186–3191.
[17] Fraker, P.J. and Speck, J.C.J. (1978) Biochem. Biophys. Res. Commun. 80, 849–857.
[18] Innis, M.A., Gelfand, D.H., Sninsky, J.J. and White, T.J., (eds.) PCR protocol, (1990) Academic Press Inc. CA.
[19] Konkel, D.A., Maizel, J.V. and Lader, P. (1979) Cell 18, 865–873.
[20] Minty, A.J., Caravatti, M., Robert, B., Cohen, A., Daubas, P., Weydert, A., Gros, F. and Buckingham, E. (1981) J. Biol. Chem. 256, 1008–1014.
[21] Misawa, Y., Tojo, A. and Shibuya, M. (1990) Biochem. Biophys. Res. Commun. 170, 39–45.
[22] Ikawa, Y., Aida, M. and Inoue, Y. (1976) Gann 67, 767–770.
[23] Hino, M., Tojo, A., Miyazono, K., Miura, Y., Chiba, S., Eto, Y., Shibai, H. and Takaku, F. (1989) J. Biol. Chem. 264, 10309–10314.
[24] Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. 162, 156–159.
[25] Scatchard, G. (1949) Ann. N.Y. Acad. Sci. 51, 660–672.
[26] Flanagan, J.G., Chan, D.C. and Leder, P. (1991) Cell 64, 1025–1035.
[27] Avraham, H., Scadden, D.T., Chi, S., Broudy, V.C., Zsebo, K.M. and Groopman, J.E. (1992) Blood 80, 1679–1684.
[28] Ogawa, M., Matsuzaki, Y., Nishikawa, S., Hayashi, S., Kunisada, T., Sudo, T., Kina, T., Nakauchi, H. and Nishikawa, S. (1991) J. Exp. Med. 174, 63–71.
[29] Welham, M.J. and Schrader, J.W. (1991) Mol. Cell. Biol. 11, 2901–2904.