Erythroid differentiation denucleation factors (EDDFs) function as intrinsic, post-erythropoietin regulators for mammalian erythroid terminal differentiation

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Abstract. Regulatory factors other than erythropoietin (Epo) dependence, that control mammalian erythroid terminal differentiation, are currently uncertain. Here we report the existence of erythroid differentiation factors in erythroid cytoplasm. Purification of these factors from cultured Friend virus anaemia (FVA)-infected mouse splenic erythroblasts was carried out using isoelectrophoresis and high performance of liquid chromatography techniques. We have identified intracellular erythroid differentiation denucleation factors (EDDFs) that were able to mediate the events of post-Epo-dependent erythroblast terminal differentiation. Purified EDDF proteins bound specifically to the enhancer HS₂ sequence of the globin gene activated the expression of haemoglobin in mouse erythroleukaemia and K562 erythroleukaemic cells and promoted them to differentiate into mature erythrocytes. EDDF proteins began to emerge at the pro-early erythroblast stages upon exposure to Epo in culture, and increased dramatically in early erythroblast stage. The dynamic of EDDF expression and its action on the key events of erythroblast differentiation and denucleation appeared to be closely consistent with its spatiotemporal distribution. These results suggest that EDDFs are the critical intracellular regulatory factors that may act as the successive regulators to Epo, responsible for the final stages of erythroid terminal differentiation.

INTRODUCTION

Erythropoiesis is the process of sequential gene expression under the orchestrated influence of multiple cellular factors such as burst-promoting factor, colony-stimulating factor (CSF-E), interleukin-3, -6 (IL-3,-6), activin A and erythropoietin (Epo), which act on different stages of

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the haematopoietic cell development in their proliferation and differentiation (Burgess 1985; Cowling & Dexter 1992; Shiozaki *et al.* 1992; Koury *et al.* 2002). It has been demonstrated that Epo is the sole regulatory factor involved in the late stages of erythroid differentiation until finally they lose their dependence at the basophil/erythroblast stage (Koury & Bondurant 1992). However, the factors in regulation of the final differentional events, of nuclear and organelle extrusion after and prior to Epo dependence, are still uncertain. Previous studies concerning the cause of denucleation have suggested that it might require interaction of extrinsic factors or accessory cell action such as with fibronectin (Patel & Lodish 1987), macrophages (Hanspal *et al.* 1998) or sinusoid endothelium (Tavassoli 1978). Gene knockout experimentation has shown that deoxyribonuclease II (DNase II)-deficient macrophages inhibit enucleation of erythroid precursors, and nucleate erythroblasts have been found in the peripheral blood of such mutant embryos (Kawane *et al.* 2001). In large-scale experiments using *ex vivo* cultures, direct contact is necessary between the adherent stromal layer and the erythroblasts for erythroid enucleation to take place (Ciarratana *et al.* 2005); however, there has been little insight into the mechanism involved. Yet, it has been illustrated that enucleation is an autonomous process (caused by intracellular regulators) that can be observed *in vitro* where accessory cells are absent (Koury *et al.* 1989; Liu & Xue 1989). The unknown intracellular regulators might be induced, upon exposure to dimethyl sulfoxide (DMSO), to redifferentiate mouse erythroleukaemia (MEL) cells (Watanabe *et al.* 1988), or might be induced to repress the vimentin gene in MEL cells (Ngai *et al.* 1984). Our previous studies have also pointed out that breakdown of nuclear anchorage vimentin initiating factor (IF) during differentiation of late erythroblasts facilitates nuclear eccentric localization and can be regarded as the initial step of denucleation (Xue *et al.* 1997). All the previously mentioned data indicate that erythroid differentiation requires induction of intracellular regulatory factors generated intrinsically or upon exposure to extrinsic cues. Epo is the principal hormone regulator of red blood cell production, survival and inhibition of apoptosis; when Friend virus anaemia (FVA) cells are cultured in the presence of Epo, a portion of the cells is able to differentiate into reticulocytes even though they have lost Epo dependence (Koury *et al.* 1984). This has been suggested to be a pathway to aberrant apoptosis (Koury *et al.* 1992) or it may logically be proposed to be regulated by unknown intracellular factors induced by Epo. In the present study, we describe data obtained from cell fusion to determine the existence of erythroid differentiation denucleation factors (EDDF) in mammalian erythroblasts. Purification of EDDFs is then described, and analyses of the spatiotemporal pattern and the role of key events in differentiation and denucleation of post-Epo-dependent erythroblasts, plus redifferentiation of erythroleukaemic cells. These results have led us to conclude that EDDFs are the critical factors beyond Epo dependence for erythroblast terminal differentiation.

MATERIALS AND METHODS

Animals and materials

Balb/c female mice of 8–12 weeks and New Zealand white rabbits (2–3 kg) were obtained from the animal centre of Chinese Academy of Medical Sciences (CAMS Beijing), housed in a specific pathogen-free environment and fed with standard rodent diet *ad libitum*. FVA (maintained by passage of infectious plasma in Balb/c mice) and the human Epo were gifts from Dr M. J. Koury (Vanderbilt University, USA). Chicken brain antisera to α -tubulin (T-9026), mouse monoclonal antiactin (A4700), human skin fibroblast vimentin antiserum (V6630), mouse monoclonal antispectrin (S3396) and fluorescein isothiocyanate (FITC)-labelled monoclonal goat

antibodies were purchased from Sigma (St. Louis, MO, USA). Chemicals such as phenylhydrazin and polyethylene glycol (PEG 1000 mw) and other reagents were also obtained from Sigma.

Cell culture

Female Balb/c mice were injected via the tail vein with 1×10^4 focus-forming units of FVA, whereas controls were injected with normal dilute serum, at 2 weeks prior to sacrifice. FVA-infected splenic proerythroblasts were isolated from the enlarged spleens and were cultured as has been previously described (Koury *et al.* 1984; Xue *et al.* 1997). Briefly, splenic tissue was minced and single cell suspensions were obtained by straining the contents through metal mesh filters and by pipetting repeatedly. FVA-infected cells were separated by velocity sedimentation at 1 g. Relatively homogenous populations of pro-erythroblasts were cultured with 0.2 U of Epo/ml in Iscove's Modified Dulbecco's Medium (IMDM) (Invitrogen, Carlsbad, CA, USA) containing 30% foetal bovine serum, 0.1% bovine serum albumin, 100 U/ml penicillin, 100 µg/mg streptomycin and 10^{-4} M α -thioglycerol, for 12, 24, 36, 48, 60 and 72 h at 37 °C in an atmosphere of 5% CO₂. Cell morphology was monitored by staining with 3,3-dimethoxybenzidine after being cytocentrifuged onto glass slides. Experimental cells were harvested at 12-h intervals for cytological study, for preparation of erythroid cell extract and for purification of EDDFs. The K562 (human erythroleukaemia) cell line was grown at 37 °C in RPMI-1640 containing 5% foetal calf serum. Friend virus-infected MEL were maintained in Dulbecco's Modified Eagle's Medium, supplemented with 15% FCS. Both cell lines were cultured at 37 °C in an atmosphere of 95% air and 5% CO₂. For induction experiments, inducers (DMSO, EDDF) were added to culture media prior to the addition of cells. Cell viability was determined by trypan blue exclusion.

Cellular extraction and purification of EDDF

Friend virus anaemia-infected cells were washed and centrifuged (1500 g, 20 min at 4 °C) with phosphate-buffered saline (PBS), and were then lysed by freezing and thawing three times at 20 °C. Mouse erythroblast cellular extract lysates (MECEL) of various stages of erythroblast differentiation were separated by centrifugation at 12000 g for 30 min at 4 °C and the supernatant was filtered through a 0.2 µm Milipore membrane (Milipore Corp. Waltham, MA). Portions of the protein were further fractionized between 50% to 75% saturation with ammonium sulphate, were dialysed and were applied for liquid isoelectrophoresis (IEF-I,II,III). Active parts were purified by high-performance liquid chromatography (HPLC) and were eluted by reversed phase (RP)-HPLC (Liu 1995) using a C18 column (Bio-Rad, Hi-pore RP304 column, 250 × 4.6 mm). EDDF fractions were routinely monitored by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining. MECEL protein samples to be tested at concentrations of 0.1, 0.25 and 0.5 mg/ml were added to the MEL cell culture system for certain periods of culture for the growth and differentiation assays. In order to isolate EDDF active protein, samples (20 µl of 1 mg MECEL) were further separated by 20% native fast gel electrophoresis (50 × 40 × 0.45 mm, Pharmacia LKB, BioTech.AB, Uppsala Sweden) and protein band strips, with their different molecular weights, were cut down for EDDF activity screening. Each protein band strip was cocultured with MEL cells (2×10^4 /ml) for 7 days and percentage was prepared of cell numbers of differentiated and denucleated reticulocytes, between protein band strips and against control gel strips (not containing MECEL). This was determined by differential cell count of differentiated and mature erythrocytes. Protein bands with apparent molecular weight (8.8 and 12 kDa) and high percentage of differentiated reticulocytes were chosen and were used as antigen foci by being injected intraperitoneally into Balb/c mice after fine mincing and were mixed with equal volumes of complete Freund's adjuvant in physiological saline. Three immunizations were carried out every 7 days. Spleen cells of the

mice were harvested and were fused with SP2/0 myeloma cells, for preparation of anti-EDDF monoclonal antibodies (Zhang 1994). Similar coculture experiments were performed with the 8.8 and 12 kDa protein band strips and with the specific antibodies, respectively, in order to confirm specific EDDF activities and inhibition by binding of their antibodies.

Histochemical and immunofluorescent microscopy

Histochemical staining for DNA, RNA, ACPase, and haemoglobin were performed according to methods described by Pearse (1960). For microtubulin, actin and spectrin, samples were fixed in 3.7% formaldehyde in PIPES/EDTA/MgSO₄/DMSO (PEMD)-PBS buffer (0.5% Triton X100/phenolphthalein monophosphate (PMP), 1% Triton X100/PBS, 1% DMSO, 1% glycerol PBS), for 30 min at room temperature. For vimentin IF staining, erythroblasts were fixed in methanol and in acetone for 7 and 3 min, respectively, at 20 °C, then were washed extensively with PBS, and permeabilized in 0.5% Triton X100 in PBS for 5 min, followed by incubation with respective antibodies and secondary FITC-conjugated goat antimouse IgG (Sigma) (Xue & Zhang 1997). Negative controls were performed using the secondary antibodies only. Cells on coverslips were observed on a laser confocal scanning microscope equipped with epifluorescence optics, serial optical section (0.6 µm); they were scanned and radiometric confocal measurement of fluorescence was performed. For rabbit antimouse mouse erythroid differentiation denucleation factor (MEDDF) antiserum, the horseradish peroxidase-labelled goat antirabbit antiserum was used as secondary antibody and diaminobenzidine (DAB) staining was employed to detect expression of target proteins.

Cybridization

Natural enucleate reticulocytes (hypoxanthine guanine phosphoribosyltransferase (HGPRT⁺)) were used as cytoplasts to fuse with HGPRT⁻ myeloma/leukaemia cells, using the HGPRT gene product as marker for cybrid cell selection. Mouse or rabbit reticulocytes were obtained by use of an anaemia model of six daily hypodermic injections of phenylhydrazine (0.1 ml of 1% per Balb/c mouse, and 0.3 ml/kg body weight of 2.5% per rabbit) as described by Bishop *et al.* 1961). The animals were bled on the eighth day; blood was washed with saline, and was centrifuged. The pellet was resuspended in Ca²⁺-free Hanks solution, and cells were counted after staining with 1% brilliant cresyl blue. Approximately 95% of the cells obtained were reticulocytes.

Fusion of mouse reticulocytes with human promyelocyte mutant cells (HL-60-AR)

Briefly, an exponentially growing HL-60-AR cell (HGPRT⁻) suspension was mixed with freshly prepared mouse reticulocytes at a ratio of 1 : 5 to 10 in Ca²⁺-free and PHA (25 µg/ml) Hanks solution at room temperature, to allow cell agglutination. They were then centrifuged and the pellet was resuspended in Ca²⁺-free medium. The cells were exposed to 0.5 ml of 45% PEG (MW 1450) for 90 s, were washed and resuspended and cultured in RPMI-1640 medium for 24 h. Appropriate cultures were then selected in hypoxanthine-aminopterin-thymidine (HAT) (containing 0.1 M hypoxanthine, 0.01 M aminopterin and 0.02 M thymidine) and HT medium. Recovered cells were maintained in RPMI-1640 medium. Cell clones were obtained from surviving cells after a period of latency of 1 to 2 months. Benzidine stain was used to identify cybrid cells. The yield of cybrid cells was about 30% after selection.

Fusion of Neo gene transferred rabbit reticulocytes and K562 cells

Using the Neo gene (from PSV2 Neo plasmid DNA) as marker, the lipofectin (Invitrogen) technique was used to transfer the Neo gene DNA to rabbit reticulocytes. K562 cells were then mixed with the reticulocytes (RRneo) at a ratio of 1 : 5 to 10. PEG was employed to promote cell fusion, and cells were selected through G418 medium (500 µg/ml) to obtain the resistant strain

of cybrid cells, K-RRneo. Clonal growth of the cybrid cells emerged on the background at about the 20th day of culture.

Northern blot analysis

Extraction of RNA was carried out according a procedure described previously (Chirgwin & Pryzbla 1979). Total RNA from various cell types was separated on agarose gel containing 1% formaldehyde, and was transferred to nitrocellulose for Northern blot analysis with specific ³²P-labelled cDNA probes. Hybridization signals were detected with the aid of a DNA detection kit (Invitrogen).

Western blot analysis

Cell lysates were prepared in sodium dodecyl sulphate (SDS) sample buffer as described previously, and then were separated by SDS-polyacrylamide gel electrophoresis (PAGE) according to the procedure of Laemmli (1970). For Western blot analysis, proteins were electrophoretically transferred to nitrocellulose, and were then blotted with various primary antibodies. After being washed, the blots were incubated with biotinylated goat antimouse IgG (Sigma). Signal was detected with a biotin-based detection system.

Southern blot analysis

Cell extracts from erythroblasts and K562 erythroleukaemia cells were separated by SDS-PAGE and were transferred to nitrocellulose as described previously. Digoxin-labelled HS2 cDNA probe (gift from Dr T. P. Liu) was hybridized and detected as described.

RESULTS

Differentiation of cybrid cells

Two different types of interspecific cell fusion models were performed in order to examine whether the erythroleukaemia/myelomal cells could be induced to recover their lost differentiation features. Results demonstrated suppression of the malignant phenotype and reappearance of terminally differentiated characteristics in all fused cells (Table 1). These displayed features

Table 1. Phenotypic characteristics of interspecific cybrid cells crossed between the anucleated reticulocytes (or nucleated erythroblasts) and the HGPRT-deficient myeloma or erythroleukaemia cell lines

Normal parental cytoplasm	Malignant parental cell line	Cybrid cell	Phenotype characters
Mouse reticulocyte (RM)	Human HL-60-AR pro-myelocytic leukaemia mutant (HL)	HL-RM cybrid	Malignancy reversion ^a expression of human haemoglobins
Neo transferred rabbit reticulocyte (RRneo)	Human K562 erythroleukaemia cell (K)	K-RRneo cybrid	Malignancy reversion ^a expression of human haemoglobins, repression of vimentin gene
Mouse B-lymphocyte (non-erythroid control)	Mouse NS-1	Lymphocyte hybridomas	Plasmacytoma (k) tumour

^aRefers to the decrease in growth rate, mitotic index and DNA synthesis, loss of colony-forming ability in soft agar medium and non-tumorigenicity in nude mice.

Figure 1. Gene expression of cybrid cells. Northern blot analysis of parental and cybrid cell RNAs to 32 P-labelled gene probes to *c-myc*, *vimentin* and β -globin. (a–b) Suppression of the *c-myc* gene in cybrid cells. Lane 1, HL-60-AR leukaemia cells; lane 2, reticulocytes; lane 3, HL-RM cybrid cells; lane 4, K562 erythroleukaemia cells; lane 5, reticulocytes; lane 6, K-RRneo cybrid cells. (c) Suppression of *vimentin* gene in cybrid cells. Lane 1, K562 cells; lane 2, reticulocytes; lane 3, K-RRneo cybrid cells. (d–e) Activation of β -globin gene expression in cybrid cells. d, lane 1, HL-60-AR cells; lane 2, HL-RM cybrid cells. (e) Lane 1, K562 cells; lane 2, reticulocytes; lane 3, K-RRneo cybrid cells (faint band). (f) Reverse transcriptase–polymerase chain reaction of cybrid cell (K-RRneo) β -globin gene RNA magnified at 158 bp. Lane 1, DNA size marker, ϕ 174/HaeIII; lane 2, RNA from K562 cells; lane 3, RNA from K-RRneo cybrid cells. (g) Blockage of vimentin gene expression in cybrid cells. Western blot analysis of cell extract lysates detected with antivimentin antibody. Lane 1, molecular weight marker; lane 2, K-RRneo cybrid cell lysate; lane 3, K562 cell lysate, and lysate digested with DNase in lane 6, showing a single band of vimentin protein at 55KDa; lane 4, rabbit reticulocyte lysate; lane 5, vimentin protein marker at 55KDa. (h) Expression of haemoglobin in cybrid cells. SDS-PAGE analysis of human β -globin gene product haemoglobin in parental and cybrid cells. Lanes 1 and 6, lysates from human erythrocytes; lanes 2 and 5, lysates from cybrid cells HL-RM; lane 3, lysate from leukaemia cell mutant cell line HL-60-AR; lane 4, lysate from mouse reticulocytes. (i) Cytological process of denucleation in cybrid cells. Microscopic observation of cybrid cell HL-RM 1–2 weeks following cybridization; 1–2, showing three cybrid cells with dark blue benzidine-stained reticulocytic components in cytoplasm and neutral red-stained host nucleus; 3–4, cybrid cells in the process of nuclear extrusion. Magnification $\times 120$.

such as decreased growth rate, lose of colony-forming ability in soft agar medium and being non-tumorigenic in nude mice (data not shown). Originally active oncogenes (*c-myc*) and vimentin IF gene were repressed (Fig. 1a,b,c), whereas the previously inactive globin genes of erythroleukaemia K562 and non-erythroleukaemia HL-60-AR cells were reactivated (Fig. 1d,e). Both cybrid cells synthesized haemoglobin of the host cell type in a specific pattern. Figure 1h shows the presence of polypeptide chains of mouse and human haemoglobins in different cybrid cells, respectively, indicating that globin genes of a non-erythroid cell type, HL-60-AR, are not

irreversibly repressed in tumour cells and can be reprogrammed upon stimulation of transacting regulatory factors. Western blot analysis revealed that the main component of vimentin in K562 cells was a single band at 55 kDa, but it was undetectable in the K-RRneo cybrids and in reticulocyte cells (Fig. 1g). These data confirm that the vimentin gene was repressed following cybridization. It is notable to point out that in cell fusion experiments, the process of nuclear pyknosis and extrusion was readily traceable by continuous microscopic observation in some of the HL-RM cybrid cells *in vitro* following cybridization (Fig. 1i), suggesting that erythroid cytoplasm may contain intrinsic factors that promote nuclear condensation and denucleation. Together, these data suggest the existence of erythroid-specific regulatory factors in mammalian erythroblasts, which may play a key role in the regulation of gene expression associated with erythroid terminal differentiation as well as inducing leukaemia cell redifferentiation.

Purification and spatiotemporal distribution of EDDFs

Based on cybridization results, we sought to isolate and purify the erythroid regulatory molecules from the cultured FVA-infected Balb/c mouse splenic erythroblasts, that had been harvested at 12-h intervals by the IEF-HPLC procedure (Table 2). Through several steps of HPLC and SDS-PAGE, we identified proteins with differentiation-inducing activity from the MECEL (Fig. 2a,b). Active proteins with molecular weight ranging from 8, 12, 14, 18 and 85 kDa were obtained (Fig. 2c) and designated as EDDFs. The EDDF proteins could bind specifically to enhancers of the HS₂ sequence of the 5' flanking regulatory elements of the β -globin gene (Fig. 2c). They were glycoproteins with features of relatively heat stable, sensitive to protease K but not to DNase and RNase. *In vitro* assays revealed that EDDFs were able to promote the positive cell number shown by benzidine staining (Fig. 2d) and to reduce the mitotic index and the cell growth rate (Fig. 2e) in a dose-dependent manner in MEL and K562 erythroleukaemia cells. MECEL isolated from intermediate erythroblasts appeared to contain the highest level of active EDDF as determined by the inhibition rate of MEL-cell proliferation growth (Fig. 2e). About 60–70% of the MEL cells became differentiated and smaller in cell size and 10–20% differentiated into reticulocytes or mature erythrocytes (Fig. 3a,b) upon EDDF stimulation, whereas over 90% of the MEL cells remained undifferentiated in the control cultures, with no EDDF, or treated with respective monoclonal antibodies (Fig. 3a). Analysis of EDDF spatiotemporal distribution in differential developmental erythroblasts cultures demonstrated that EDDFs began to emerge intracellularly at pro- and early-erythroblast stages upon exposure to Epo in culture (Fig. 3c). Its expression was very low in pro-erythroblasts, but increased markedly thereafter. EDDFs activated the expression of the globin gene at the early-erythroblast stage, in which cell differentiation appeared to lose its dependence on Epo (Fig. 3c). Kinetic analysis showed that

Table 2. Summary of purification of EDDF

Step	Total protein (mg)	Yield (%)	Purification (fold)
Spleen extract	83000	100	1.0
Amonium sulphate	32000	97	2.5
IEF(I)	1200	42	29
IEF(II)	65	34	430
IEF(III)	12	25	1700
HPLC (gel filtration)	4.2	16	3200
Centricon-10	1.8	13	5900
RP-HPLC (c18)	0.8	8.2	8500

Figure 2. Purification and biological activity assay of EDDF. (a) Purification of EDDF by HPLC chromatography. Erythroid lysate sample with biological activity from third round IEF applied to the column and eluted at room temperature with flow rate of 1.0 ml/min fraction 'a' showing EDDF activity. (b) SDS-PAGE analysis of fraction 'a' EDDF in Figure 2a, showing a single band at 8.2 kDa molecule weight. (c) Identification of HS₂ binding proteins. Southern blot analysis of cellular extract lysates of mouse mid-late erythroblasts and K562 cells by using digoxigenin-labelled HS₂ as probe. M protein markers; lanes 1, 2, K562 cell lysates after EDDF (50 ng/ml) treatment; lanes 3, 4, K562 cell lysates before EDDF treatment; lanes 5, 6, cell lysates of mouse mid to late erythroblasts. 1X and 2X, protein concentration of 20 µg and 40 µg/ml, respectively. (d) EDDF-induced haemoglobin expression. Benzidine staining of MEL cells before (2d-1) and 5 days after EDDF (50 ng/ml) treatment (2d-2). Magnification ×300. (e) Effects of MECEL (mouse erythroblast cellular extract lysate) on the growth curve of MEL cells. Comparison of the effects of varying concentration (0.1 mg, 0.25 mg and 0.5 mg/ml) of MECEL from intermediate erythroblasts with those from late erythroblasts and reticulocytes, on the growth curves of MEL cells (6×10^4 cells/ml) for 7 days *in vitro*, using 1.8% DMSO as controls. Each datum represents the $\bar{X} \pm \text{SD}$ of three experiments.

EDDF was active throughout the whole terminal differentiation period, with an active peak at the intermediate-to-late erythroblast stage, which preceded the sudden decline of nuclear anchoring vimentin IF and nuclear condensation (Fig. 3c). Figure 3c also illustrates that EDDF action on the key differentiation events of post-Epo-dependent erythroblasts were compatible

with its spatiotemporal contribution. Moreover, in view of this spatiotemporal distribution between Epo and EDDFs, it seems likely that EDDFs take over the relay baton from Epo and carry on as successive regulators responsible for the final stages of erythroid differentiation.

Potential role of EDDF in erythroid terminal differentiation

Based on the sequencing of purified EDDF proteins, novel gene clones of EDDF such as MEDDF (murine EDDF), HEDDF (human EDDF), HEDRF-1 (human EDDF-1) and HEDRF-2 (human EDDF-2) have been cloned in our laboratory and registered in GenBank. All of their gene products bind specifically to the HS₂ enhancer of the β -globin gene and all are haematopoietic-tissue specific. However, they were variously expressed in a stage-specific manner and function as the post-EPO-regulator family for mammalian erythroid terminal differentiation. Here we have characterized the MEDDF clone (GenBank No: AF 060220) in detail, which represents the gene relevant to nuclear condensation and extrusion. It was found to contain full-length 505-bp nucleotides with an open reading frame, encoding a protein of 102 amino acids, with molecular weight deduced to be 11.8 kDa. The MEDDF protein was expressed successfully and with high efficiency (40%) in *Escherichia coli* through standard recombinant DNA expression system. A high titre of antiserum was developed after inoculating the protein as antigen to rabbit. The immunocytochemical study demonstrated that the MEDDF protein was barely detectable in the cytoplasm of pro-/early erythroblasts, but it increased markedly during intermediate erythroblast stages, and transferred from the cytoplasm to the nuclei of the late erythroblasts. These stained dark brown in colour and the stage was simultaneously followed by a series of nuclear pyknosis events and the process of denucleation (Fig. 4a,b,c), indicating that the MEDDF is an inducing molecule for the commitment of the final steps of erythroid terminal differentiation. Northern blot analysis revealed that the gene products were haematopoietic-tissue specific (Fig. 4d). In an attempt to examine whether MEDDF existed or was expressed in non-mammalian (*Avis*) erythroblasts, chicken bone marrow erythroblast RNA and cells were utilized. Northern blot and immunofluorescence analyses illustrated that MEDDF gene products could readily be detected in mammalian (mouse) samples, but not in non-denucleated chicken erythroblasts (Fig. 4e,f,g,h). These results indicate that there was no homology of EDDFs existing in chicken erythroid cells. The results are consistent with the fact that unique erythroid denucleation occurs exclusively in mammalian erythroblasts but not in those of non-denucleated or non-mammalian erythroblasts; this might imply that EDDFs may be generated evolutionarily in mammalian erythrocytes during phylogenesis as regulators of the final stages of erythroid terminal differentiation.

DISCUSSION

The reticulocyte as a reliable natural cytoplasm model for cybridization

The cybridization technique has been used extensively in the study of cytoplasmic factors and to examine a variety of biological problems. Previous studies have reported that artificially isolated cytoplasts can suppress tumourigenicity when fused to recipient malignant cells (Howell & Sager 1978). However, other cybrid studies on cytoplasmic suppression have been generally inconclusive (Shay & Werbin 1988) because (a) artificial cytoplasts usually have suffered damage to cell organelle constituents such as mitochondrial DNA and (b) the enucleation procedure can hardly obtain unsubdued putative cytoplasmic suppressors or regulators and consistent pure cytoplasts. In the present study, we have for the first time chosen naturally denucleated mouse reticulocytes as the cytoplasts to fuse directly with the HGPRT⁻ human promyelocyte cell

Figure 3. The effects of MECEL on MEL cell differentiation. (a) Histogram of the effects of MECEL, protein band-strips, and respective monoclonal antibodies on MEL cell differentiation. Depicts the effects on MEL cells (2×10^4) compared to cocultured with MECEL (0.5 mg/ml, panel III), protein band strips contain MECEL of 8.8 KDa (panel VI) and 12 KDa (panel IV) and respective monoclonal antibodies (panels VII and V) for 7 days *in vitro* on the percentage of

Figures 4. Intracellular transfer of MEDDF protein involved in nuclear condensation and denucleation.

DAB immunocytochemical staining by using rabbit anti-MEDDF sera. (a) Light yellow stained MEDDF protein in pro-erythroblast cytoplasm; (b) transfer of intensified DAB stained protein from cytoplasm to nuclei of early/mid erythroblasts; (c) dark brown staining of MEDDF protein on the condensed and extruded nuclei of late erythroblasts. Magnification $\times 400$; (d) tissue and stage specificity expression of MEDDF products. Northern blot analysis of MEDDF mRNA to ^{32}P - MEDDF probe showing tissue specific expression in bone marrow, spleen and mid-late erythroblasts, (1) proerythroblasts, (2) mid-late erythroblasts, (3) brain; (4) heart; (5) liver; (6) spleen; (7) lung; (8) kidney; (9) bone marrow; (10) muscle; (11) testis. (e–h) Species specificity expression of MEDDF. Northern blot (e, f) and immunofluorescence (g, h) analysis of the expression of MEDDF in mouse and chicken bone marrow erythroblasts. (e) RNAs from mouse peripheral erythrocytes (lane 1) and bone marrow (lane 2) showing a positive band for MEDDF, but were negative for RNAs from those of chicken erythrocytes (lanes 3, 4), respectively; (f) GAPDH; (g), mouse bone marrow erythroblasts showing positive staining of MEDDF protein; (h) chicken bone marrow erythroblasts showing negative reaction.

Figure 3. Legend continued

benzidine-positive differentiated cells, reticulocyte/mature cells and undifferentiated cells. Using 1.8% DMSO (panel II) as positive control and gel strips (no MECEL, panel VIII) as negative controls. (b) Morphological characteristics of MEL cells cocultured with MECEL for 7 days. (1) MEL cells before coculture, stained; (2) MEL cells cocultured with 1.8% DMSO (positive control) and (3) MEL cells cocultured with MECEL (0.5% mg/ml), most of the cells differentiated into reticulocytes and mature cells. Benzidine stained bar = 10 μm ; (c) Relative spatiotemporal curve of EDDF during murine erythroblast terminal differentiation. Illustrates EDDF dynamic changes relevant to erythroblast intracellular biochemical events, nuclear condensation and denucleation. Note that EDDF emerged at the pro-early erythroblast stages and a surge appeared at the mid-late erythroblast stages – concurrent with the sudden drop of vimentin IF and prior to nuclear condensation and extrusion. EDDF was assayed by cell extract lysates from cultured FVA-infected mouse erythroblasts harvested at 12-h intervals. The effect of EDDF was justified by inhibition rate on MEL cell growth and differentiation of *in vitro* assay. Data points represent mean of triplicate measurements. E, erythrocyte; E-very, early erythroblast; Int-ery, intermediate erythroblast; P-ery, Proerythroblast; L1, L2, L3, substages of late erythroblast; R, reticulocyte.

mutant (HL-60-AR) or fusion of rabbit reticulocytes, after transfer of the Neo gene into human K562 erythroleukaemia cells. Both techniques gave rise to higher fusion rates (30–40%) after HAT or G418 medium selection, and more pure cybrid cells (with positive benzidine staining) than those with only 5% fusion rate of artificial enucleation techniques. Further advantages are (1) the natural denucleate reticulocyte contains cytoplasmic organelles such as mitochondria, Golgi complex, endoplasmic reticulum, lysosomes and a variable number of molecules such as ferritin and enzyme proteins (cholinesterase, catalase and ubiquitin pathway enzymes) and siderosomes for vigorous survival; (2) the reticulocyte contains a series of long-lived mRNAs and retains mono- or polyribosomes and other components of protein synthesis or degradation machinery, which can continuously to work in protein metabolism and regulate gene expression (Wefes *et al.* 1995); (3) the reticulocyte is rich in the HGPRT enzyme, which can be used as genetic marker for selection of viable cybrid cells. By employing reticulocytes as the parental cytoplasts to fuse with human leukaemia cells, we have confirmed the existence of EDDF regulatory factors in erythroid cytoplasm and the effect of EDDFs on the malignant phenotypes of myeloma and erythroleukaemia. This novel cybridization model also provides evidence that the anuclear reticulocyte allows expression of the transferred foreign gene and can acquire a heritable trait for the cybrid cells. So, this is a new approach and an experimental model that may aid further elucidation of the regulatory effects as well as the mechanism of action of the cytoplasmic factors in tumour cell malignancy.

The role of EDDF in post-Epo-dependent erythroblast differentiation

It has been shown that FVA-infected erythroid cells bear a surface expressing Epo receptors. The number of Epo receptors increases to a maximum in Epo-dependent cells, but progressively declined during the process of terminal differentiation, to nearly complete loss of the receptor at the basophilic/erythroblast stage – that is, when the cell begins haemoglobin synthesis (Koury *et al.* 1992). After that, the factors that regulate the program of post-Epo-dependent erythroblast differentiation are poorly understood. It has been proposed that a putative regulatory factor would be induced in cells upon exposure to Epo, which may be responsible for post-Epo erythroid cell differentiation, although no such regulators had been reported yet. In the present study, through cybridization experiments, we have confirmed the existence of intracellular regulatory factors in erythroid cytoplasm. Furthermore, we have identified specific regulatory factors (EDDFs) by the techniques of cellular extraction and protein purification, and found that EDDFs play a pivotal role in the key terminal differentiation events of post-Epo-dependent erythroblasts. EDDF proteins can bind specifically to the HS₂ sequence of the globin gene, and they might acts as transacting regulatory factors in cybrid cells to activate the expression of the previously inactive globin gene, even in non-erythroid cell types, which coincides with the findings of Baron (1986) of globin gene expression in heterokaryons. On the other hand, these cells suppressed the expression of the *c-myc* oncogene and *vimentin IF* gene following induced erythroid cell differentiation; that is consistent with the reports of Westin *et al.* (1982) and Ngai *et al.* (1984), respectively. These results suggested that the cytoplasm of reticulocytes might contain a complex of transacting regulatory factors in the programming of post-Epo-dependent genes' on–off expression. Following cybridized cell fusion, erythroleukaemia/myeloma cells deficient in terminal differentiation factors were now able to be induced to recover their lost differentiatonal phenotypes and to express a variety of cellular functions. A series of cytological processes and of nuclear condensation, eccentric location and denucleation were also readily observed in some cybrid cells, after incorporation with the reticulocyte component. It is worth mentioning that the timing of EDDF protein emergence began just before the basophilic/erythroblast stage, at which erythroid cells lose their Epo dependence and begin haemoglobin synthesis. Therefore, EDDF

action on the integral events of differentiation and denucleation that occur to post-Epo-dependent erythroblasts corresponds well to EDDF's spatiotemporal contribution. Thus, it is conceivable to assume that EDDFs might be induced by Epo, and function as successive regulatory factors for post-Epo-dependent erythroblast terminal differentiation. According to Koury and Bondurant (1990), Epo is required continuously for erythroblast survival during terminal differentiation. When deprived of Epo in culture, FVA-infected cells die. Here we show that besides cell survival maintenance, Epo might exert a further important role through inducing endogenous EDDF in erythroblasts. In addition, MEDDF protein has been demonstrated in dynamic transfer from the cytoplasm in the mid-erythroblast, being intranuclear in the late erythroblast; this is followed by a series of cytological processes of nuclear condensation and denucleation. Furthermore, Northern blot analysis revealed that MEDDF mRNA was tissue- and species-specific, with abundant expression only in mammalian (mouse) bone marrow and spleen (mid-to-late erythroblasts), but not in *Avis* (chicken) erythroblasts. Similarly, in immunofluorescence assays, by using the anti-EDDF antibody, it was also shown that only mouse erythroblasts expressed EDDFs but not chicken; demonstrated by positive fluorescent staining. These data indicate that there are no homologies to EDDF in the *Avis* family, so we suggest that EDDFs might be an evolutionarily product generated only in enucleated mammalian erythroblasts during phylogenesis, in adapting to their microcirculation development. Further work is required in order to illustrate the molecular pathway of EDDF action plus studies on EDDF target cell receptors. The phylogenetic derivation of EDDFs as well as their generation as evolutionarily products, and how they may cause chromatin condensation before enucleation, are all key problems to be considered as parts of further studies in our laboratory. Nuclear condensation has been well established as a vital biological phenomenon, and is critical in erythroblast denucleation events. It has been shown that both the histone and non-histone protein content of chromosomes is involved in the condensation process. Also, according to recent reports, the condensin complex, cohesin and topoisomerase II, may play important synergistic roles in chromosome condensation/decondensation during the cell cycle (Anderson *et al.* 2002; Hirano 2002; Cuvier & Hirano 2003). However, in mammalian erythroid cells, nuclear pyknosis is irreversible chromatin condensation and this leads to enucleation; its total mechanism is still mysterious, awaiting further investigation.

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