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Erythroid differentiation regulator (EDR), a novel, highly conserved factor I. Induction of haemoglobin synthesis in erythroleukaemic cells

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Abstract

In serum-free WEHI-3 supernatants an activity was detected inducing haemoglobin synthesis in human and murine erythroleukaemia cell lines. The absolute numbers of benzidine-positive cells induced with either DMSO or WEHI-3-conditioned medium were comparable. Terminal differentiation was not observed. An expression library from WEHI-3 RNA aided by PCR cloning revealed an open reading frame corresponding to a 209 amino acid protein. This was 100% identical to a sequence from human stimulated peripheral blood mononuclear cells. In contrast to human RNA, mouse RNA exhibited multiple bands of pre-mRNA in Northern blots. The gene was provisionally termed erythroid differentiation regulator (*edr*).

In mammalian cells EDR is mostly expressed as a 56 kDa dimer showing higher activity than the recombinant monomer. The activity profile is bell-shaped. Expression was observed in many normal mouse tissues, yet in haematopoiesis it was largely confined to $CD34^+$ cells. It was enhanced by a series of stimuli such as phorbol ester, and transformed cells generally showed a higher level of EDR expression than normal ones. The protein is localized at the inner side of the cytoplasmic membrane and is released in part via vesicles. In view of the broad range of EDR-expressing tissues the function obviously exceeds haemoglobin synthesis induction. Involvement in cell survival and growth control has been observed and will be dealt with in detail elsewhere.

Keywords: Differentiation; Erythroleukaemia; Haemoglobin synthesis; RNA splicing; Vesicles

1. Introduction

In vitro maturation of erythroid cells derived from CFU-E largely depends on erythropoietin (Epo) and insulin-like growth factor I which account for approximately 70% of the stimulating activity, while the remaining 30% may be contributed by a variety of unidentified serum factors [1]. BFU-E colony development, on the other hand, is enhanced by a large number of more or less well-defined factors. Among these are kit-ligand (KL) [2,3], GM-CSF and IL-3 [4], IL-6 [5], IL-9 [6,7], IL-11 [8], erythroid-potentiating activity [9], membrane-bound burst-promoting activity [10], human erythroid burst-stimulating activity [11] and thrombopoietin [12]. Activin A [13] has been shown to act on BFU-E indirectly via mononuclear or stromal cells [14]. The actual contribution of the various factors in vivo is unknown and may vary considerably under different conditions. Most of these factors are not erythroid specific and in one and the other case the physiological significance for

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erythropoiesis is not well established. Further and as yet unidentified activities can be assumed to exist. Even proteases may have a stimulatory effect on erythropoiesis [15,16].

In the present study a novel factor will be presented detected in serum-free supernatants of WEHI-3 cells due to its haemoglobin synthesis-inducing property in human K562 and murine erythroleukaemia (MEL) cell lines. The nucleotide sequence is the same in man and mouse, and it is expressed in many different normal murine tissues. Properties and special features of the factor at the RNA and protein level, its expression and the haemoglobin synthesis-inducing activity will be described. In a following study (manuscript in preparation) it will be shown that this activity provisionally termed erythroid differentiation regulator (EDR) is an autocrine stress-related factor for cell survival and growth control.

2. Results

2.1. Haemoglobin-inducing activity

WEHI-3 conditioned medium (WCM) generated by incubation of cells in serum-free medium (SFM) showed haemoglobin-inducing activity in F4N, B8/3 and K562 cells. Only weak effects were observed when WEHI-3 cells instead were left in normal medium containing 10% fetal calf serum (FCS) (data not shown). In MEL up to 30% of cells became benzidine-positive by day 4 (Fig. 1A), and in K562 cells up to 45% (data not shown). This is roughly half of the maximum value obtained with chemical compounds like DMSO in these MEL cells and obtainable with activin A or haemin in the case of K562 cells. However, the absolute numbers of benzidine-positive cells on day 4 were in the same order of magnitude for both DMSO and WCM as shown in Fig. 1B for F4N cells: DMSO induced 8.8×10^5 benzidine-positive cells/ml, and 3 different WCM preparations $8.5 \pm 5.4\times10^5$/ml. In B8/3 4.5×10^5 benzidine-positive cells/ml were induced by DMSO, and $5.1 \pm 1.7\times 10^5$ by 3 different WCM preparations (data not shown).

Many WCM batches exhibited a bell-shaped dose–response profile (Fig. 1A). Peak concentration and peak height of a batch depended not only on WCM properties but also on conditions of the erythroleukaemia cells such as seeding cell density and age of cells in culture. Most activity was released from WEHI-3 cells within the first 12 h after transfer to SFM (Fig. 1A). Activity was also found in supernatants of NIH-3T3 cells in medium containing 2% FCS (data not shown), and in supernatants of the human bone marrow stromal cell line L88/5 after irradiation (Fig. 1C). Properties of individual FCS batches in growth medium turned out to be of significance for the later activity yield in SFM from WEHI-3 cells suggesting that the specific mRNA was formed at least in part prior to transfer of cells into SFM. Fractionation of WCM on a Sephacryl S 300 column revealed 1 major peak of α-globin mRNA-inducing activity (Fig. 2, upper panel). An analysis of benzidine-positive B8/3 cells after incubation with fractions of WCM indicated an apparent M_r between 40 and 60 kDa (Fig. 2, bottom panel). There was one further peak with 10% WCM in fraction no. 27 of Fig. 2,

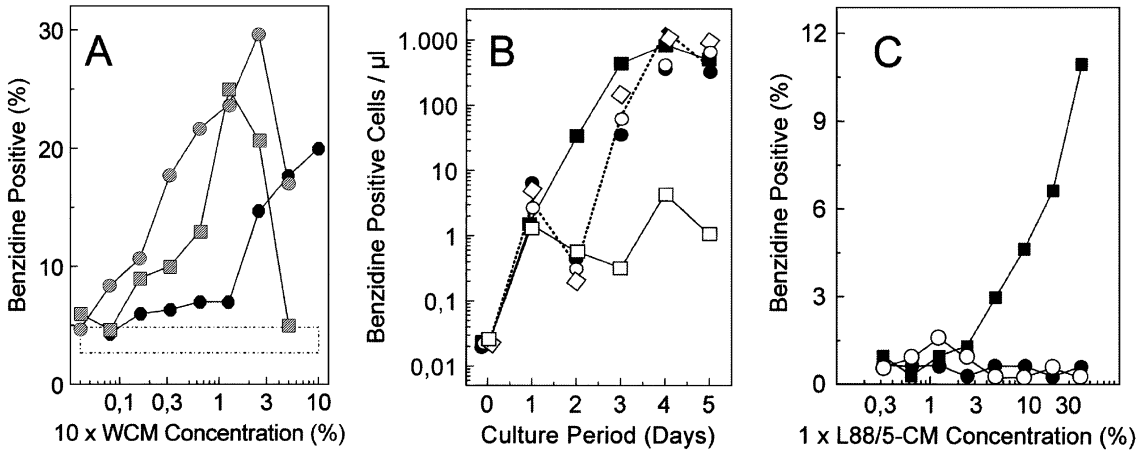

Fig. 1. Effects of conditioned media on MEL cells. Panel A: dose–response curves with 3 batches of WCM, measured by the percentage of benzidine-positive B8/3 cells. Solid octagons, batch of WCM used in most experiments as a positive control; shaded symbols, WCM generated by cultivation of WEHI-3 cells in SFM for 12 h (shaded boxes) or 3 days (shaded circles); dotted rectangle, control (mean values ± 1 SD) with growth medium alone. Pilot control experiments with various concentrations of 10×SFM versus growth medium alone had revealed no systematic differences. Panel B: absolute numbers of benzidine-positive cells per ml of F4N cell cultures incubated with either 1.2% DMSO (solid boxes), with 20% of 10×WCM (3 batches of WCM: open diamonds, open and closed circles), or growth medium as control (open boxes). Panel C: L88/5 human stromal cells were grown to confluence and then irradiated. Cultures were continued in SFM/growth medium for 5 days and conditioned media were harvested. Symbols: closed boxes, 15 Gy irradiation and continuation in SFM; open circles, sham irradiation and continuation in SFM; closed circles, sham irradiation and continuation in growth medium.

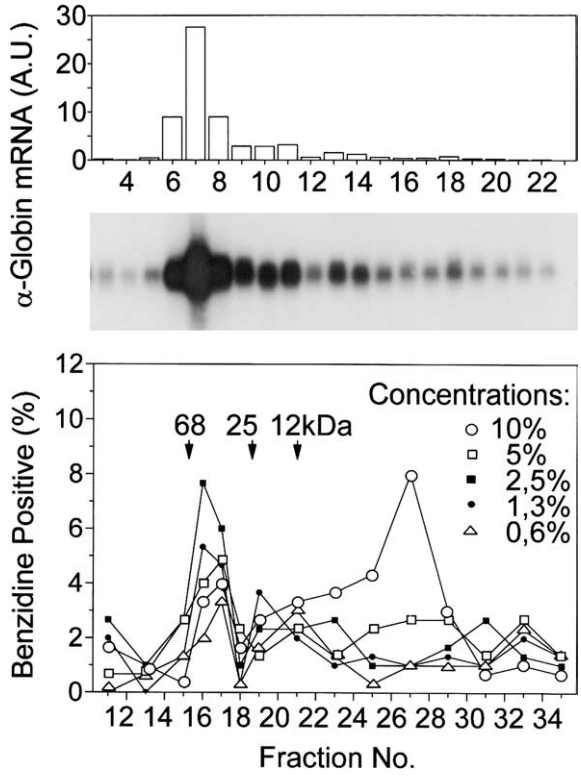

Fig. 2. Fractionation of 25×WCM on a sepharose gel and incubation of B8/3 cells with individual fractions. Upper panel (densitogram) and middle panel (autoradiograph), Northern blot analysis performed with total RNA of B8/3 cells incubated with 25% (final concentration) of fractions for 4 days. A fragment of murine α-globin cDNA was used as a probe. Bottom panel: incubation of B8/3 cells with varying concentrations of individual fractions of a WCM batch different from the one of above. Determination of percentages of benzidine-positive cells after 4 days of incubation. Fraction sizes varied between both experiments. Panels have been aligned in such a way that the albumin peaks and thereby also the closely located fractions with the highest activities are positioned one below the other.

bottom panel, caused by a low-molecular weight constituent and not seen in the upper panel which was not further pursued. Various cytokines were tested with respect to haemoglobin synthesis-inducing potential for MEL cells. KL, TGF-β_1, IL-6, IL-3, TNFα and LIF were completely negative, while Epo had only an effect at doses exceeding 2 U/ml (data not shown). Activin A [17] was unlikely to account for the major effect, since the homodimer exhibits an M_r of only 25 kDa [18].

2.2. *Studies of* edr *mRNA*

An expression library from WEHI-3 mRNA was constructed. After transfection of a total of 25,000 clones into COS-1 cells one supernatant inducing haemoglobin synthesis in B8/3 cells was detected. The positive clone (DY-8) showed an activity slightly less than that of the standard WCM used as a positive control throughout. The insert was 715 bp in length and had an open reading frame (ORF) of 534 bp calculated from the first in-frame ATG (EMBL Acc. No. AJ007909). The corresponding gene was provisionally termed *edr* (erythroid differentiation regulator).

Using the insert of DY-8 as a probe the WEHI-3 as well as an NIH-3T3 cDNA library were screened. Among 40 positive clones of variable lengths no insert exceeded 1400 bp. An insert of 1361 bp (clone HA-15/2, similar to EMBL Acc. No. A93591) was used to study *edr* in a Northern blot of WEHI-3 RNA before and after transfer of cells to SFM (Fig. 3). A whole panel of bands was found irrespective of applying cytoplasmic or total RNA. In total RNA it included several bands of high molecular weight. There was a moderate increase in *edr* mRNA accumulation 1 h after transfer of cells to SFM.

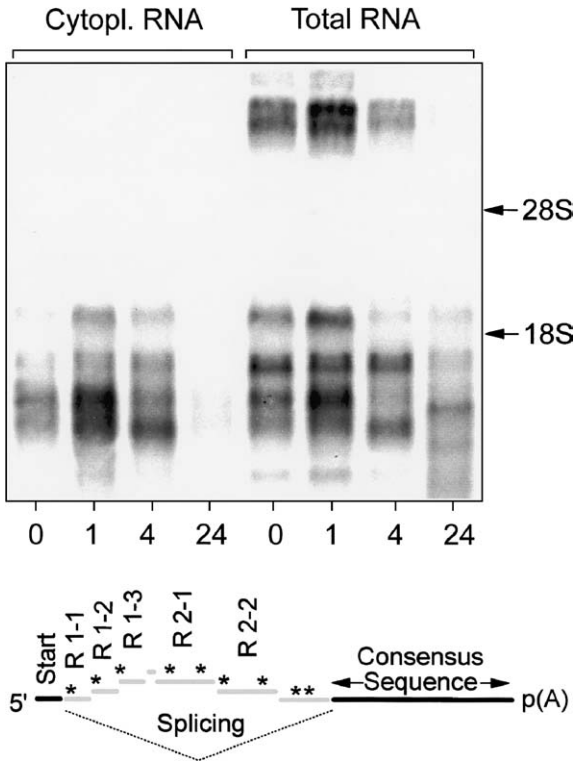

Fig. 3. Northern blot of WEHI-3 RNA and analysis of sequence diversity. Upper panel: Northern blot of WEHI-3 RNA performed with cytoplasmic RNA (left-hand side) or total RNA (right-hand side). Cells were harvested prior to, and 1, 4, and 24 h after transfer from growth medium to SFM. After hybridization with a probe of 2×10^6 cpm/ml from HA-15/2 for 24 h, washes were performed at high stringency: 3×10 min 2×SSC/0.1% SDS at 22 °C; 30 min 0.5×SSC/0.1% SDS at 60 °C, 30 min 0.2×SSC/0.1% SDS at 60 °C, and 15 min 0.1×SSC/0.1% SDS at 60 °C. A film was exposed to the filter for 3 days. Lower panel: nucleotide structure of *edr* clones consisting of a 5′ start sequence, a middle part with numerous repeats, and a 3′ consensus sequence. Variable numbers of repeats were found in individual clones and thus are to be found in the various *edr* bands in Northern blots. Overall, 3 81 bp repeats (R1-1 to R1-3) and of 2 180 bp repeats (R2-1 to R2-2) along with 2 further unique stretches containing numerous stop codons (symbolized by asterisks) have been detected in the middle part. In the final translatable *edr* sequence all of these are spliced out.

A sequence analysis of various positive clones revealed a 3′ consensus sequence of 632 bp preceded by a variable, AT-rich middle part consisting of variable numbers of 81 and 180 bp repeats thus resulting in variable overall sequence lengths, and a short 5′ consensus stretch containing the ATG codon (Fig. 3). These clones do not have a through-ORF since both 81 and 180 bp repeats harbour several stop codons. It was detected, however, in RT-PCR amplificates of WEHI-3 as well as normal CBA spleen RNA in which the 5′ ATG-containing consensus stretch coding for the 3 amino acids MLG followed by the first 6 nucleotides of repeat 81/1 coding for LV were directly spliced to the 3′ consensus sequence (EMBL Acc. No. AJ539223). This sequence was obtained by analysing individual bands out of a whole ladder, which had been RT-PCR-amplified with one and the same primer pair. Furthermore, an identical nucleotide sequence of the ORF resulted from RT-PCR-amplification of human peripheral blood mononuclear cell (PBMC) RNA by using a forward primer spanning the junction of the 5′ and 3′ consensus regions.

The insert of clone HA-15/2 served as a probe for analysing various RNA samples from murine tissues and cell lines in a Northern blot (Fig. 4). Among normal mouse tissues thymus showed the strongest bands. In 8 of 8 normal mouse tissues analysed *edr* bands were detectable (data not shown). These were placenta, liver, brain, lung, intestine, spleen, bone marrow and thymus. According to our nucleotide sequence studies, none of the many and variable bands in Northern blots corresponds to the final translatable *edr* transcript. Fig. 4 suggests that cell lines in general accumulate more *edr* mRNA than primary tissue. As an exception, no expression at the total RNA level was detectable in M1 cells. Quite different results were obtained in the human system. A Northern blot analysis of various human haematopoietic primary cells and cell lines using total RNA and HA-15/2 as a probe revealed only diffuse and weak bands above 1600 bp. Also at the poly(A)$^+$ level studied in some of the samples no bands were detectable below 1600 bp (data not shown). RNA sequences corresponding to that one of clone HA-15/2 were classified as *edr* pre-mRNA.

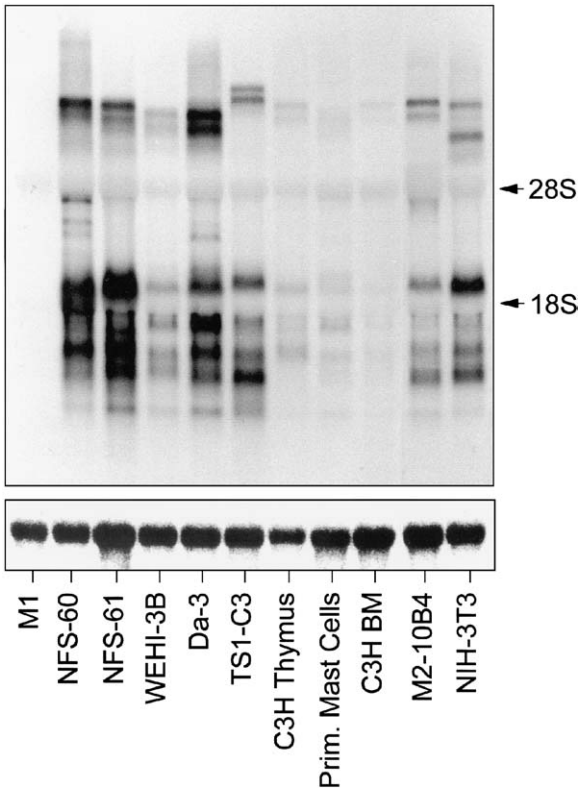

Fig. 4. Northern blot analysis of total RNA from various murine sources. Primary mast cells were generated with a purity exceeding 98% by cultivating Balb/c mouse bone marrow in the presence of rmu IL-3 for 6 weeks. After hybridization with a probe of 2×10^6 cpm/ml from clone HA-15/2 for 24 h, washing was performed at high stringency: 3×10 min $2 \times$ SSC/0.1% SDS at 22 °C, 30 min $0.5 \times$ SSC/0.1% SDS at 60 °C, 30 min $0.2 \times$ SSC/0.1% SDS at 60 °C, 20 min $0.1 \times$ SSC/0.1% SDS at 60 °C. Film was exposed to the filter for 10 days. Abbreviation: BM, bone marrow.

2.3. Studies of EDR protein

The EDR protein sequence deduced from the ORF consists of 209 amino acids. It has been unknown so far and does not contain any known motifs. Recombinant EDR produced in *Escherichia coli* shows an apparent M_r of 28–29 kDa (data not shown). By using the monoclonal antibody 8A12 in Western blots of normal and abnormal human and murine cells and tissues a major band is detectable at 56 kDa and 2 minor bands at 28 and 112 kDa likely to represent EDR dimer, monomer, and tetramer, respectively (Fig. 5A). In many instances only the dimer was found. Between human and murine samples there were no obvious differences.

In normal human blood and bone marrow cells appreciable EDR bands were found only in the CD34$^+$ fraction (Fig. 5B, No. 4), exactly in 4 out of 9 cases. Three of these had been pretreated with G-CSF. It is worth noting that lymphoblastoid cell lines consisting of transformed B-cells [19,20] and used as positive controls in many immunoblots (Figs. 5B, C) always gave strong EDR signals. In contrast, the LD fractions in Fig. 5B, Nos. 3 and 5 containing approximately 20% of normal B-cells did not show any signal, whereas white blood cells (WBC) from a B-CLL patient were positive (Fig. 5C, No. 2). This supports the notion that corresponding to the RNA data also at the protein level, transformed cells exhibit more EDR accumulation than primary cells. In a CML case there was some EDR positivity in whole WBC (Fig. 5C, No. 3). However, as with normal blood cells, the CD34$^+$ LD fraction showed the strongest band (Fig. 5C, No. 5).

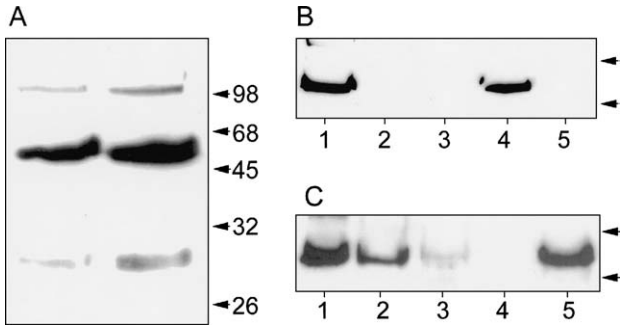

Fig. 5. Immunoblots of EDR in murine and human samples using Mab 8A12. Panel A: fractionation and blot of 10 μl (left) and 15 μl (right) of a primary murine lymphoma lysate. Panel B: leukapheresis material from a healthy donor pretreated with G-CSF. Lane 1, positive control with 1×10^5 lymphoblastoid cell line (LCL) cells; lane 2, 2×10^5 cells of total white blood cells (WBC); lane 3, 2×10^5 cells of LD fraction; lane 4, 1×10^5 $CD34^+$ cells; lane 5, 2×10^5 $CD34^-$ LD cells. Panel C: in each lane lysates from 2×10^5 cells were loaded. Lane 1, positive control with LCL cells; lane 2, WBC from a case of B-CLL (WBC count 50,000/μl); lane 3, WBC from a case of CML (WBC count 18,000/μl); lane 4, LD fraction of CML WBC; lane 5, $CD34^+$ fraction of CML WBC. Material in 3, 4, and 5 was derived from the same CML patient. Upper and lower arrows in panels B and C refer to 68 kDa and 45 kDa MW markers, respectively.

Within cells EDR is distributed at the inner side of the cytoplasmic membrane (Fig. 6A, B). In B8/3 as well as in other cultured cells blebs and cytoplasmic fragments filled with EDR can be observed at the cell surface and also sometimes distant from cells suggesting EDR release. In contrast to the results of EDR-negative LD fractions of PBMC in Figs. 5B, No. 5 and 5C, No. 4, Fig. 6B indicates significant EDR expression in cord blood T-cells after incubation with IL-4 and IL-7. Comparable activation at the RNA level has been observed in mouse spleen cells stimulated with either PHA, PMA or anti-T-cell receptor antibody (data not shown). Both B8/3 (Fig. 6A) and stimulated cord blood T-cells (Fig. 6B) show a consistent phenomenon of EDR disappearance at the site of close contact between cells.

Two different cDNA sequences were cloned into the mammalian expression vector pRc-CMV, one starting with the first in-frame ATG of clone DY-8 (534 bp *edr*), and one in which the DY-8 sequence starting with position 55 was connected to an ATG (636 bp *edr*). The constructs were transfected into CX-2 cells and the supernatants collected. Fig. 7A shows that activities from both constructs are comparable to standard WCM. The activity of the 636 bp construct exceeds that of the 534 bp construct by a factor of 2.7. The bell-shaped profile is reminiscent of WCM in Fig. 1A. In K562 cells haemoglobin induction by the 534 bp *edr* construct was only slightly inferior to that by activin A (data not shown). Fig. 7B depicts the activity profile of G-EDR. While the maximum activity of recombinant supernatants from CX-2 cells amounted to a factor of 3.5 above background, G-EDR reached only a factor of 2. Very similar results were obtained with His-EDR, and both recombinant *E. coli* products showed a bell-shaped profile.

Proof for the identity with EDR of the haemoglobin synthesis-inducing factor in WCM was obtained by

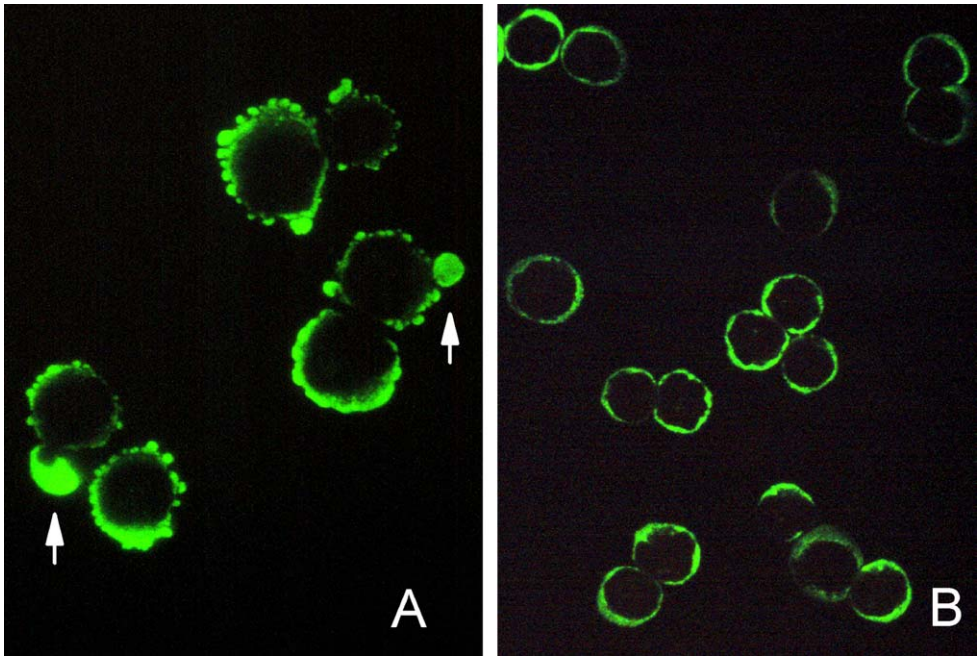

Fig. 6. Immuno-staining of cytocentrifuge preparations of murine and human cells with Mab 8A12. Panel A: MEL B8/3 cells. Note the large blebs and cytoplasmic fragments on some of the cells seeming about to be released (arrows). Panel B: human cord blood T-cells. After a 7-day incubation of mononuclear cells with IL-4 and IL-7 T-cells were separated by cell sorter using an anti-CD3 antibody and lysed.

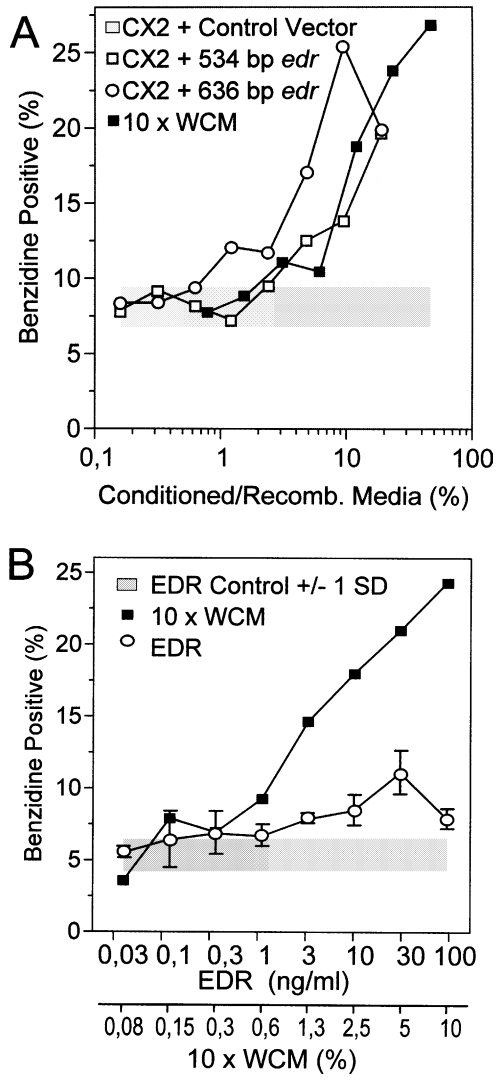

Fig. 7. Effect of recombinant EDR on B8/3 cells. Panel A: two sequences of different lengths derived from clone DY-8 were cloned into the Rc-CMV vector and transiently transfected into CX-2 cells. Supernatants were tested in B8/3 cells (day 4). Shaded area is mean value ± 1 SD of B8/3 cell benzidine positivity after incubation with corresponding dilutions of supernatant from control vector-transfected CX-2 cells. Panel B: B8/3 cells were incubated for 3 days with recombinant *E. coli*-derived EDR having 1 additional glycine at the N-terminus (G-EDR). For every experimental point 3×300 B8/3 cells were counted. Error bars indicate means ± 1 SD. Shaded rectangle refers to mean value ± 1 SD obtained by incubating B8/3 cells with corresponding dilutions of a preparation derived from a vector without insert.

incubation of WCM with the anti-EDR monoclonal antibody 1D10, which resulted in a concentration-dependent neutralization of the WEHI-3 activity (Fig. 8). At low antibody concentrations the percentage of benzidine-positive cells exceeded that of WCM in the presence of control isotype. This repeatedly made observation has to be studied further. In order to exclude that the antibody effect was primarily directed against EDR released from B8/3 cells, also other differentiation

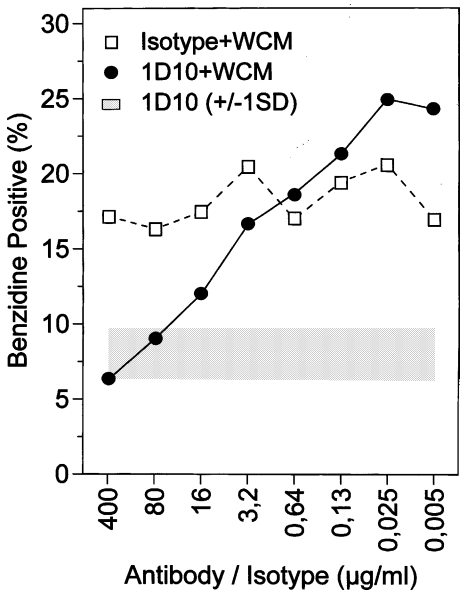

Fig. 8. Anti-EDR antibody neutralizes haemoglobin synthesis-inducing activity of WCM. B8/3 cells were incubated with constant amounts of 5% 10×WCM and varying concentrations of anti-EDR Mab 1D10 or a control isotype. Presented are 1 out of 4 experiments with the same outcome.

inductors were tested in place of WCM. These were 1% DMSO, 2.5 µM HMBA, 100 ng/ml activin A, and 10 U/ml rh Epo. With none of these inductors the antibody showed a statistically significant effect (data not shown).

2.4. Stimulation of EDR transcription and release

Transfection of the repeat-containing *edr* sequence of clone HA-15/2 into COS-1 cells did not result in an activity increase in supernatants (Fig. 9A). However, in an attempt to enhance EDR release by the addition of phorbol ester following transformation the activity rose by a factor of 1.9 in the control, and in the *edr*-containing vector experiment by 3.1 ($p < 0.01$) (Fig. 9A). Post-translational EDR phosphorylation by protein kinase C was unlikely to account for this effect, since in vitro phosphorylation of rEDR with protein kinase C did not alter the activity, and the same was true with casein kinase II (data not shown). One effect involved in protein kinase C action and likely to be also relevant here is the release of vesicles [21,22]. Vesicles containing haemoglobin synthesis-inducing activity are indeed released by WEHI-3 cells (Fig. 9B), and dimerized EDR has been detected in Western blots of the vesicle fraction (data not shown). In addition to an enhancement of factor release, transcriptional activation is assumed to occur, since the addition of PMA to short-term cultures of mouse spleen cells induced an *edr* mRNA increase within 1 h with a maximum at 4 h (data not shown).

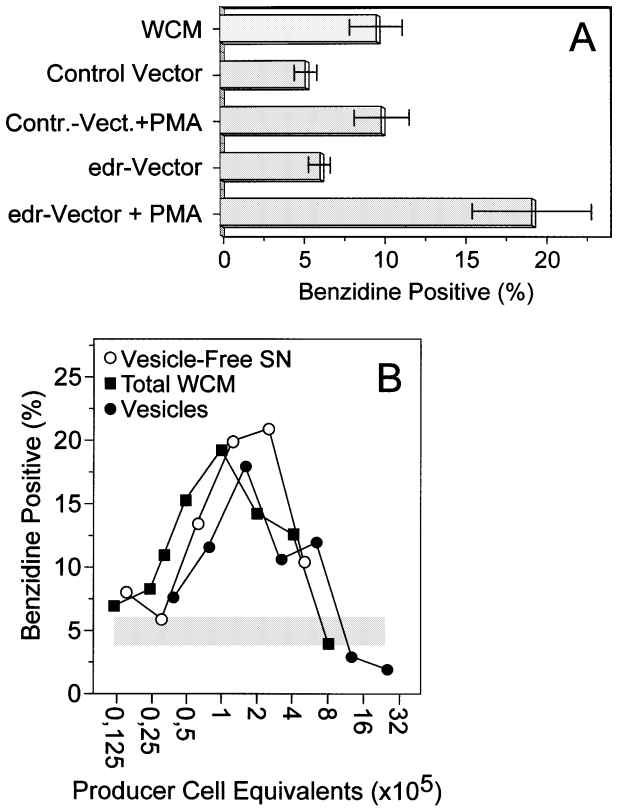

Fig. 9. PMA increases EDR activity in conditioned media. Panel A: the repeat-containing insert of clone HA-15/2 in vector pXPRS$^+$ was transiently transfected into COS-1 cells. Supernatants obtained in the presence/absence of 50 ng/ml PMA were tested in B8/3 cells and benzidine-positive cells were counted on day 4. Error bars: mean values ± 1 SEM from results with 6.4, 3.2, and 1.6% of supernatants, respectively. The mean WCM concentration in this experiment was only 3.7%. Panel B: vesicles isolated from WCM show EDR activity on B8/3 cells (day 4). Activities were related to the numbers of contributing producer cells (see Section 4). As shedding of vesicles is stimulated by phorbol ester this offers one possible explanation for the activity increase by phorbol ester in Panel A. The shaded rectangle represents mean value ± 1 SD of benzidine positivity after incubation of B8/3 cells with growth medium only.

2.5. Protein properties

Due to a high proportion of 15.8% of arginine EDR is quite basophilic reaching a theoretical pI of 11.76. A signal peptide was not detected. The molecule is predominantly hydrophilic with a hydrophobic N-terminus which prevented expression in *E. coli* unless covered by a $(His)_6$ or TEV protease recognition sequence. According to the N-end rule [23], the estimated half-life in mammalian reticulocytes amounts to 30 h. However, following the stability calculations by Guruprasad et al. [24] it is classified as unstable. Except for serum-free supernatants from WEHI-3 and *edr*-transfected CX-2 cells it has not been possible by Western blots to detect EDR in cell-conditioned media (data not shown). On the other hand, EDR was detected by Western blot in a batch of normal FCS by agitating 5 ml in an 80 cm^2 culture flask for 1 h, then rinsing off all FCS with phosphate-buffered saline (PBS) and collecting the protein sticking to the plastic bottom by lysis buffer (data not shown). This emphasizes a high degree of stickiness.

3. Discussion

An activity has been detected in supernatants of murine WEHI-3 and NIH-3T3 cells as well as human L88/5 cells inducing haemoglobin synthesis in human K562 and murine Friend erythroleukaemia cell lines. Origin from, and action on, both human and murine cell lines suggested complete species cross-reactivity. The stimulatory effects of serum deprivation or irradiation indicate that stress conditions enhance production and/or release. Gel chromatographic analysis of WCM showed one major activity peak exhibiting an apparent M_r, which rendered activin A unlikely to represent the major candidate under study. Other cytokines could be excluded since the MEL cell lines we used were unresponsive or at least largely unresponsive to them.

The percentage of benzidine-positive cells induced by WCM was less than by other erythroid differentiation inducers. However, the overall yield of haemoglobinized MEL cells was comparable between WCM and DMSO suggesting that WCM was less toxic than DMSO. On the other hand, in contrast to DMSO terminal differentiation with a significant percentage of cells attaining the appearance of orthochromatic erythroblasts and extruding their nuclei was observed neither by recombinant CX-2 supernatants nor by WCM. WCM (Figs. 1A, 9B) as well as rCX-2-CM (Fig. 7A) and rEDR (Fig. 7B) showed bell-shaped activity profiles.

Northern blots of murine RNAs revealed a series of *edr* bands which human RNAs did not show, yet sequences likely to represent the translated *edr* version and identical by 100% between mouse and man were amplified by RT-PCR from both RNAs. It is concluded that *edr* is a gene highly conserved between man and mouse and associated with differences in mRNA processing between species with gross pre-mRNA accumulation in murine cells even in the cytoplasmic compartment. Accumulation of intron-containing pre-mRNA has been associated so far primarily with transformation [25]. *edr* shows that the normal system may also be involved. Whether only nuclear *edr* pre-mRNA is further processed or also the cytoplasmic component has not been investigated. The translatable form of *edr* mRNA was undetectable in Northern blots using either total or poly(A)$^+$ RNA of either mouse or man, however, it could be demonstrated by PCR. This suggests that it is not accumulated to a larger extent but rather spliced out of precursor RNA upon demand and immediately inserted into the translational process.

Proof of mRNA by PCR, but not by Northern blot, was also encountered in a study of various secreted cytokines in unstimulated human bone marrow stromal cell lines [26]. Taking into account that Northern blots of murine RNA detect *edr* pre-mRNA, the diversity of bands in Fig. 4 is less confusing. Interestingly, consistent bands were detectable between different tissues and cell lines from the same mouse strain, however, not between the same tissue from different strains.

A size of 28 kDa of rEDR compares well with the monomeric EDR band detected in Western blots (Fig. 5A). By gel chromatography the major WCM activity was located between 40 and 60 kDa (Fig. 2). This corresponds to the major and frequently single EDR band of 56 kDa in Western blots suggesting that EDR in vivo exerts its activity mainly as a dimer. Therefore, the question arose whether the monomeric rEDR has to dimerize to become fully active. Two different rEDR refolding procedures and use of 2 different more or less complete EDR sequences were unsuccessful in resolving this question revealing only little difference regarding haemoglobin synthesis-inducing activity. The question regarding dimerization and resulting activity remains open. A positive relationship still seems likely, since other conditions of post-translational modification in cells such as phosphorylation by protein kinase C or casein kinase II were approached experimentally without significant activity changes.

EDR is localized at the inner side of the cytoplasmic membrane (Fig. 6). Fig. 6A suggests that it is released from B8/3 cells via blebs likely to represent a mechanism of rapid release. Fig. 9A further suggests that in WEHI-3 cells the release is under the control of protein kinase C. EDR is released from these cells in part via vesicles retaining biological activity, and in part secreted or shed in a form not precipitable by ultracentrifugation (Fig. 9B).

Two questions arise regarding the wide EDR distribution among normal tissues: (1) Is haemoglobin synthesis induction a function of such importance that so many cell types produce this inductor? (2) Why do B8/3 cells respond to a factor provided from outside that they themselves produce and obviously also release? Regarding the first question, experiments to be reported elsewhere have revealed a stress-related involvement of EDR in cell survival and growth control (manuscript in preparation). This will shed more light on the widely distributed occurrence of EDR and its major function. Concerning the second question, the ability of B8/3 cells to respond to recombinant EDR is obvious according to Fig. 7B. However, the suitability of EDR molecules released from B8/3 cells for stimulation may be questioned. According to Fig. 6A, Edr is released from these cells by large cytoplasmic particles and blebs. In contrast to the fraction of vesicles from WEHI-3 cells (Fig. 9B), the fraction of vesicles from B8/3 cells obtained by ultracentrifugation did not contain Edr as judged by immunoblotting (data not shown). We therefore assume that Edr is released from B8/3 cells predominantly by way of larger aggregates from which single molecules or smaller groups of molecules do not go into solution due to the stickiness of EDR and which therefore are unsuitable for eliciting cellular responses.

4. Materials and methods

4.1. Cell lines

The following lines were obtained from the American Type Culture Collection (Rockville, MD, USA): WEHI-3, K562, NIH-3T3, M1 and COS-1. Cell lines NFS-60, NFS-61 [27] and DA-3 [28] were a kind gift from J. Ihle, St. Jude's Hospital, Memphis, TN, USA. Cell line TS1-C3 [29] was obtained from L. Hültner, GSF, and murine erythroleukaemia (MEL) cell lines F4N [30] and B8/3 [31] were obtained from W. Ostertag, Heinrich Pette Institut, Hamburg, Germany. Cell line M2-10B4 [32] was received from C. Eaves, Terry Fox Institute, Vancouver, Canada, and CX-2 [33] was a gift from G. Multhoff, GSF. The human bone marrow stromal cell line L88/5 had been established in this institute [34].

4.2. Growth media

If not specified otherwise, cells were cultured in RPMI-1640 medium with 2 mM L-glutamine, 100 U/ml of penicillin and streptomycin, and 10% of pretested FCS, termed growth medium in the following text. Instead of RPMI-1640, COS-1 cells were cultured in Dulbecco's MEM with 4.5 g/l glucose. Serum-free medium (SFM) for the generation of WCM consisted of RPMI-1640 containing 0.1% bovine serum albumin (BSA), 2 mM L-glutamine, 20 U/ml of penicillin and streptomycin, 32 mg/ml iron-saturated human transferrin, 10^{-5} M 1-α-dipalmitoyl lecithin, 2×10^{-5} M oleic acid, and 2×10^{-5} M cholesterol [35] (all chemicals from Sigma–Aldrich, Taufkirchen, Germany).

4.3. WCM generation

WEHI-3 cells were kept in growth medium in logarithmic growth, then transferred and cultured in SFM at 1×10^{6}/ml for 0.5–3 days. The supernatants were centrifuged at $1600\times g$ and 4 °C for 10 min and subsequently concentrated 10-fold in an Amicon (Witten, Germany) concentrator using a YM10 membrane (nominal M_r limit 10 kDa). After sterile filtration the samples were aliquotted and frozen at −20 °C. They were active for several years with only moderate activity loss. For gel chromatography 25-fold concentrated WCM was separated on a Sephacryl S 300 column

(Pharmacia Biotech, Freiburg, Germany) in PBS. As M_r standards BSA (68 kDa), chymotrypsinogen (25 kDa) and cytochrome *c* (12.5 kDa) were applied.

Vesicles shed from WEHI-3 cells were prepared from fresh WCM centrifuged at $1600\times g$ and 4 °C for 20 min. The supernatant was run in an ultracentrifuge at $100,000\times g$ and 4 °C for 2 h. The resultant supernatant was considered "vesicle-free" and harvested, while pellets from several tubes were resuspended in PBS, collected and run again in small conic tubes as above. Thereafter they were dissolved in a small volume of PBS and designated "vesicles". In order to compare the activities from total WCM, vesicles and vesicle-free supernatant, total volumes of the fractions were related to the cell numbers applied for their generation. Thereby, the activity at any experimental point calculated as percentage of total activity could be expressed in terms of producer cell number.

4.4. Differentiation induction and scoring of benzidine-positive cells

K562, F4N or B8/3 cells were washed once in PBS, resuspended in growth medium and seeded either in 25 cm^2 flasks at densities between 20,000 and 50,000/ml in the presence or absence of conditioned media or an inducer, or in 96-well plates at densities of 10,000 to 20,000/ml in 100 μl of total volume. For scoring of the percentages of benzidine positivity after 3–5 days, 5–10 μl of cell suspension from individual wells were pipetted into fresh microtiter plates together with fresh growth medium to make 100 μl. Five microlitre of a working solution containing *N*,*N*,*N*′,*N*′-tetra-methyl-benzidine (Sigma–Aldrich) were added, and the positive, clearly green staining cells were scored in the wells among a total of 300 cells using a Leitz Diavaert inverted microscope. The tetra-methyl-benzidine stock solution was prepared by dissolving 10 mg in 10 ml of 12% acetic acid. At the time of use, 35 μl of this solution were mixed with 35 μl of isopropanol and 5 μl of hydrogen peroxide (working solution).

4.5. RNA preparation and Northern blot analysis

Total RNA was prepared using the acid phenol method [36], and cytoplasmic RNA according to Sambrook et al. [37]. Fifteen micrograms of RNA per lane were separated on a 2.2 M formaldehyde–1.2% agarose gel and subsequently blotted onto Hybond-N membranes (Amersham–Buchler, Braunschweig, Germany). ^{32}P-labeled probes were prepared using a random labeling kit (Boehringer, Mannheim, Germany). After hybridization X-ray films were exposed to the radioactive filters, while for quantitative assessment Fuji Imaging plates were exposed and processed in a Phospho-Imager (Fuji Photo Film Co. Ltd., Japan). A 516 bp cDNA fragment of mouse α-globin mRNA used as a probe had been previously cloned out of a mouse spleen cDNA library. For standardization of sample quantities a 6.6 kbp EcoRI cDNA fragment of murine rRNA was applied (kindly provided by I. Grummt, DKFZ Heidelberg, Germany).

4.6. Reverse transcriptase-PCR (RT-PCR)

Transcripts from human PBMC and from WEHI-3 cells were RT-PCR amplified. Prior to lysis and total RNA preparation WEHI-3 cells in logarithmic phase of growth were incubated with 10 ng/ml PMA (Sigma–Aldrich) in growth medium for 30 min. Human PBMC were incubated with 5 ng/ml PMA and 1 μg/ml phytohaemagglutinin (PHA) (Biochrom AG, Berlin, Germany) in growth medium for 24 h. RT reactions of RQ1 DNase-treated (Promega, Heidelberg, Germany) total RNA were performed using Omniscript RT (Qiagen, Hilden, Germany). Due to a GC prevalence in *edr* cDNA best PCR results were obtained with the Advantage-GC 2 kit including 0–0.5 M GC-Melt (Clontech, Heidelberg, Germany). To PCR-amplify finally processed human or murine *edr* transcripts the same reverse primer: 5′-TTATTGAGGGGGGGCATTTCTG-3′ was used. A forward primer: 5′-GGAATGCTGGGACTTGTACGTC-3′ was applied in the case of human *edr* which yields 1 major band of 633 bp and includes 3 bp of the promoter region. For murine *edr* the forward primer 5′-GACCGTGCGGACTTAAGATGG-3′ was chosen yielding several amplificates of which one band of 700 bp was isolated after fractionation in low-melting point agarose and re-amplified. It contains 70 bp of the promoter region. PCR conditions were: 3 min at 94 °C, 10 cycles at 94 °C for 30 s, 65 °C for 30 s and 72 °C for 2 min. The 15–25 ensuing cycles were identical to the previous ones except for a decrement of 0.3 °C per cycle from the annealing temperature. A total of 35 cycles was run for primary amplifications, of 25 cycles for re-amplifications, and was concluded by a 5 min extension at 72 °C.

4.7. Expression cloning

For construction of an expression library WEHI-3 cells were serially harvested at densities between 5 and 10×10^5/ml from cultures containing growth medium. Cells were lysed for RNA preparation and in parallel transferred to SFM for WCM generation. The lysate of cells, which in parallel yielded the most active WCM, was selected for library construction. Poly(A)$^+$ RNA was isolated using the PolyATract kit from Promega. As a prokaryotic–eukaryotic shuttle vector the 3.753 bp vector primer pXPRS$^+$ from USB, Cleveland, Ohio, USA was used. In this plasmid gene expression in eukaryotic cells is driven from the early SV40 promoter.

It further contains an SV40 polyadenylation site [38]. The vector-primer pXPRS $^+$ and a cDNA synthesis kit provided by USB were applied according to the manufacturer's indications. Highly competent DH5 bacteria (1×10^9 clones/μg DNA) prepared according to Inoue et al. [39] were transformed with the plasmids and amplified once. Plasmids from this amplification step were run uncut on a low melting point agarose gel for exclusion of inserts of less than 500 bp. The larger ones were again transfected into highly competent DH5 bacteria. These were plated onto round 81 mm Hybond-N filters on LB-agar-containing Petri dishes at a density to give 500 clones per filter. After preparation of replica filters and regrowth of the bacteria on these filters the clones were scraped off for plasmid DNA isolation and transfection into COS-1 cells. Transfection experiments were performed in duplicate.

COS-1 cells were transiently transfected using the DEAE-dextran method [40]. Briefly, after 30 min of incubation with 5% DEAE-dextran and 500 ng DNA per 35 mm culture plate, and after incubation with 80 μM chloroquine for 2.5 h, a 3 min incubation with 15% HEPES-buffered (p_H 7.1) glycerol was performed. Subsequently growth medium was added for 72 h and, in turn, tested with B8/3 cells for haemoglobin-inducing activity. In these evaluations 1 WCM batch generated in a larger quantity served as a positive control throughout. For stable transfection into COS-1 and CX-2 cells the 5.446 bp Rc/CMV prokaryotic–eukaryotic shuttle vector (Invitrogen, The Netherlands) was applied in combination with LipofectAMINE (Life Technologies, Eggenstein, Germany).

4.8. Cell separation

Normal human bone marrow aspirates from donors of allogeneic transplants were obtained with informed consent. Low-density (LD) cells (<1.077 g/ml) were separated by Percoll (Serva, Heidelberg, Germany) and subsequently washed twice in PBS containing 2% BSA and 0.6% (w/v) tri-sodium citrate dihydrate (Merck, Darmstadt, Germany). CD34$^+$ cells were enriched using Dynabeads-CD34 (Deutsche Dynal, Hamburg, Germany) according to the manufacturer's protocol.

4.9. Recombinant EDR

rEDR was generated in *E. coli* by cloning PCR-derived *edr* sequences into the *N*-$(His)_6$-containing pQE-30 vector (Qiagen). Either sequence AJ007909 (EMBL data bank) from positions 74 to 688 (for generating His-EDR), or sequence AJ539223 (EMBL data bank) from 71 to 700 (for G-EDR) were used. In the latter case, the recognition sequence of TEV protease [41] was inserted 5′ of the ATG initiation codon by using the forward primer: 5′-AATGGATCCGAGAATCTGTACTTCC-AGGGAATGCTGGGACTTGTACG-3′. The reverse primer in both cases was: 5′-TGTAAGCTTATTGAG-GGGGGGCATTTCTG-3′. As control for the recombinant protein an empty pQE-30 vector was always processed in parallel. *E. coli* strain M15 (Qiagen) was used for transformation. Purification of the recombinant protein was performed by binding the 6×His tags to columns of Ni-Nitrilotriacetic acid (Ni-NTA) agarose (Qiagen) and washing the columns as recommended. His-EDR was refolded on the columns according to the method by Holzinger et al. [42] and eluted. Washing, refolding and elution volumes were kept identical for EDR and the empty vector control. Eluates were dialysed against PBS for 2 days in the cold and the protein concentrations determined using the BCA technique.

For G-EDR preparation the material was eluted unfolded from the column and injected into 10 volumes of refolding buffer [43]. The final concentrations in this buffer were: 1.5 M urea, 150 mM NaCl, 20 mM NaH_2PO_4, 4 mM GSH, 0.4 mM GSSG, p_H 7.9. After a 1 h incubation at room temperature EDR was enriched by another Ni-NTA column, washed, and eluted in 150 mM NaCl, 20 mM NaH_2PO_4, 50 mM EDTA, 1 mM 2-ME p_H 7.9. The material was then dialysed against PBS for 2 days at 4 °C. Subsequently, the protein was digested at 30 °C with TEV protease (Invitrogen) for 4 h by applying 1 unit of TEV protease for each 3 μg of EDR protein. The flow-through of this material from a 3rd Ni-NTA column containing EDR with 1 additional N-terminal glycine (G-EDR) was collected and diluted with PBS to 10 ng/μl, aliquotted, lyophilized and stored at −80 °C. At the time of use it was dissolved with distilled water to make 10 ng/μl and further diluted with PBS according to the experimental protocols.

4.10. EDR visualization

Two monoclonal anti-EDR antibody were generated in rats by using a C-terminal EDR peptide of 73 amino acids for immunization according to standard protocols. One antibody termed 8A12 (rat IgG2a kappa) showed superior sensitivity in Western blots and immunofluorescence but recognized only the denatured EDR protein, whereas the other one termed 1D10 (rat IGg2a kappa) was a neutralizing antibody. For polyacrylamide gel electrophoresis on a 14% Tris–Glycine gel $1-4\times10^5$ cells per lane were dissolved in a buffer consisting of 8 M urea, 1 M NaCl, 0.1 M NaH_2PO_4, 0.01 M Tris–Cl pH 8.0, 15% (v/v) glycerol and 1% Triton X-100 and sonicated. They were briefly boiled in additionally 0.2 M 2-ME and 0.025 M SDS (all chemicals from Sigma–Aldrich). After fractionation gels were transferred to nitrocellulose membranes. These were blocked with nonfat dry milk (10%, w/v) in PBS and then incubated

with either a 1:1 dilution of the 8A12 hybridoma supernatant or a 40 μg/ml dilution of the purified 8A12 monoclonal antibody in 10% nonfat dry milk. The secondary peroxidase-conjugated goat anti-rat antibody was purchased from Jackson Immuno Research Laboratories (West Grove, PA, USA). Bound antibody was detected using enhanced chemiluminescence (Pierce, Rockford, IL, USA).

For in situ staining of EDR in cytocentrifuged cells these were fixed in methanol at −20 °C for 10 min. After 3 washes in PBS/0.02% Tween20 cells were incubated with the 8A12 hybridoma supernatant diluted 1:10 in PBS/0.1% BSA at room temperature for 60 min. The secondary antibody was a DTAF-conjugated F(ab′)2 fragment of a donkey anti-rat IgG (Jackson Immuno Research Lab.) which was incubated with the cells in a 1:25 PBS/0.1% BSA dilution for 60 min. After 3 washes as above the cells were embedded in Mowiol 4-88 (Sigma–Aldrich).

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