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Differentiation of embryonic stem cells into adipocytes in vitro

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SUMMARY

Embryonic stem cells, derived from the inner cell mass of murine blastocysts, can be maintained in a totipotent state in vitro. In appropriate conditions embryonic stem cells have been shown to differentiate in vitro into various derivatives of all three primary germ layers. We describe in this paper conditions to induce differentiation of embryonic stem cells reliably and at high efficiency into adipocytes. A prerequisite is to treat early developing embryonic stem cell-derived embryoid bodies with retinoic acid for a precise period of time. Retinoic acid could not be substituted by adipogenic hormones nor by potent activators of peroxisome proliferator-activated receptors. Treatment with retinoic acid resulted in the subsequent appearance of large clusters of mature adipocytes in embryoid body outgrowths. Lipogenic and lipolytic activities as well as high level expression of adipocyte specific genes could be detected in these cultures. Analysis of expression of potential adipogenic genes, such as peroxisome proliferator-activated receptors γ and δ and CCAAT/enhancer binding protein β, during differentiation of retinoic acid-treated embryoid bodies has been performed. The temporal pattern of expression of genes encoding these nuclear factors resembled that found during mouse embryogenesis. The differentiation of embryonic stem cells into adipocytes will provide an invaluable model for the characterisation of the role of genes expressed during the adipocyte development programme and for the identification of new adipogenic regulatory genes.

Key words: Mouse embryonic stem cell, Adipocyte differentiation, Peroxisome proliferator-activated receptor

INTRODUCTION

Adipose tissue allows for triacylglycerol storage in periods of energy excess and the subsequent use of triacylglycerol stores during energy deprivation. Modification of the lipogenic/lipolytic balance as well as an increase in the adipose cell precursor population lead to obesity (Ailhaud et al., 1992). Key events in the programme of adipocyte differentiation have been identified largely through analysis of the differentiation process of immortalized preadipose cell lines. Gene markers for the preadipose and adipose stages have been defined, as have *trans*-acting factors involved in the coordinate expression of genes of terminal differentiation (Cornelius et al., 1994; MacDouglad and Lane, 1995). However, the gene(s) that commits progression from the multipotent mesodermal stem cell to the adipoblast stage of development has not yet been identified. Nor have the factors involved in self-renewal of adipoblast stem cell precursors.

It was shown several years ago that treatment of 10T1/2 mesodermal stem cells with 5-azacytidine leads to the activation of regulatory genes that commit the cells to the adipocyte, myocyte and chondrocyte lineage (Taylor and Jones, 1979). Although this model resulted in the identification of the muscle regulatory genes of the MyoD bHLH-leucine zipper transcription factor family (Olson and Klein, 1994) it has not yet permitted the identification of the master gene(s) that controls adipocyte commitment. One reason could be the low frequency of differentiation of treated cells towards the adipocyte lineage. Other cell culture models containing pluripotent mesodermal stem cells able to give rise to adipocytes have been established from tumors and SV40-transformed cells (Darmon et al., 1984; Kellerman and Kelly, 1986), or from somatic cells (Gimble, 1990). In order to develop an alternative model for studying commitment and early differentiative events in adipogenesis, we have investigated the capacity of pluripotent embryonic stem (ES) cells to undergo adipocyte differentiation.

ES cells are derived from the inner cell mass of 3.5 day blastocysts (Evans and Kaufman, 1981; Martin, 1981). ES cells can be maintained in an undifferentiated state in vitro and manipulated by various genetic techniques. They can then be reintroduced back into mouse blastocysts where they will contribute to all cell lineages of the developing mouse (reviewed by Smith, 1992). In vitro, ES cells have been shown to differentiate spontaneously into various lineages such as extra-embryonic yolk sac, cardiac, haematopoietic (Doetschman et al., 1985), skeletal muscle (Rohwedel et al., 1994) and epithelial (Bagutti et al., 1996). Differentiation events similar to those in vivo can be obtained in cell culture using ES cells (Keller, 1995). It has previously been reported, from morphological observations, that ES cells could give rise to adipocyte-like cells (Field et al., 1992). However, spontaneous commitment of ES cells into the adipocyte lineage is rare. For using ES cells

for studying early differentiative events in adipogenesis it was essential: (i) to characterize in more detail the ES cell-derived adipocyte-like cells; and (ii) to determine conditions of culture that favor the adipocyte differentiation of ES cells at a high rate. These steps have been achieved and results are reported in this paper. Our results indicate that retinoic acid (RA) is important for the early events of the adipocyte development programme in ES cells since pretreatment of differentiating ES cell-derived embryoid bodies (EBs) with RA for a short period of time resulted in adipogenesis at a high level. In contrast, addition of potent activators of peroxisome-proliferator-activated receptors (PPARs) and adipogenic hormones only during early differentiative steps did not commit ES cells into the adipocyte lineage. Studies of the expression of *PPARδ* and *PPARγ* genes in differentiating EBs suggest that adipogenesis of ES cells mimics closely the in vivo adipocyte developmental programme. The culture protocol we have devised provides a powerful system for investigating the first steps in adipogenesis both in vivo and in vitro, and a route to the isolation and characterization of the master gene(s) for adipogenesis.

MATERIALS AND METHODS

ES culture and differentiation of embryoid bodies

Embryonic stem cell lines used throughout the study were: ZIN 40 (Mountford and Smith, 1995), E14TG2a (Hooper et al., 1987) and CGR8 (Mountford et al., 1994). These cell lines are feeder-independent cell lines and were cultivated in the undifferentiated state on gelatin-coated plates. Cells were free of mycoplasma contamination and were regularly checked for. Cells were maintained in Glasgow MEM/BHK 21 medium containing 0.23% sodium bicarbonate, 1× MEM essential amino acids, 2 mM glutamine, 1 mM pyruvate, 100 μM 2-mercaptoethanol, 10% (v/v) fetal calf serum (S. A. Dutscher, France). This medium is referred as cultivation medium. Differentiation-inhibiting activity/leukemia-inhibitory factor (100 units/ml) was added to maintain the pluripotent undifferentiated state (Smith, 1991).

For differentiation ES cells were cultivated in aggregates termed embryoid bodies (EBs). Hanging drops containing 10^3 cells in 20 μl cultivation medium were maintained for 2 days on the lids of bacteriological dishes filled with phosphate-buffered saline. The embryoid bodies formed were then transferred into bacteriological plates and maintained for 3 days in suspension in cultivation medium supplemented either with 0.1% DMSO or with all-*trans* retinoic acid (RA) or with other compounds when indicated. Medium was changed every day. EBs were maintained two days more in suspension in cultivation medium then were allowed to settle onto gelatin-coated plates in the presence of differentiation medium. This medium consists of cultivation medium supplemented with 85 nM insulin, 2 nM triiodothyronine (T_3) and 10% selected fetal calf serum (Seromed, France). Medium was changed every two days.

RNA analysis and DNA probes

RNA was prepared and analyzed as previously described (Dani et al., 1989). Quantification of the hybridization signal was performed using a PhosphorImager apparatus (Fujix Bas 1000) coupled to the MacBas ver2.x bio-imaging analyser. The C/EBP β cDNA was isolated from MSV/C/EBP β plasmid (provided by S. L. McKnight, Tularik Inc. South San Francisco), the PPARδ/FAAR cDNA from pSG5-FAAR plasmid (from P. Grimaldi, University of Nice-Sophia Antipolis, France), the PPARγ cDNA from PPARγ2/SPORT plasmid, the adipsin cDNA from pAD20 plasmid and the ALBP cDNA from pAL422 plasmid (from B. M. Spiegelman, Dana-Farber Cancer Institute, Boston, Massachusetts).

RESULTS

Differentiation of ES cells into adipocytes at a high efficiency is induced by all-*trans* retinoic acid

Retinoic acid (RA) is a critical regulatory signal molecule in developmental process. In vitro, it has been reported that RA influences in a time- and concentration-dependent manner the efficiency and pattern of differentiation of ES cells (Wobus et al., 1994). We therefore investigated the capacity of ES cells to undergo adipocyte differentiation following exposure to RA and subsequent culture in medium permissive for adipogenic differentiation. The protocol is described in Materials and Methods and represented schematically in Fig. 1. After a period of one week in suspension ES cells formed embryoid bodies (EBs) as previously reported (Doetschman et al., 1985). The EBs were then plated in medium containing adipogenic hormones known to be essential for terminal differentiation of preadipose cells (Ailhaud et al., 1992). Spontaneously beating cardiomyocytes appeared in the dense centre of attached EBs as early as 1 day after plating (day 8 after EB formation). Fibroblast-like cells and various unidentified cell types grew out over the following 2-3 weeks. After 10-15 days clusters of cells filled with lipid droplets appeared. Fig. 2A shows a representative EB outgrowth stained with oil red 0, a specific stain for triglycerides. A large adipocyte colony which developed in the outgrowth of the aggregate could be detected. Three feeder-independent ES cell lines were used throughout the study. One of these, named ZIN40, is a marked ES cell line which has been generated by gene trapping and gives ubiquitous nuclear expression of β-galactosidase in undifferentiated ES cells and differentiated derivatives in vivo and in vitro (Mountford and Smith, 1995). Detection of a β-galactosidase activity in the nucleus of adipocytes is shown in Fig 2B.

To investigate the requirement and the specificity of RA treatment for the induction of the adipocyte programme of ES cells, embryoid bodies were treated between the 2nd and the 5th day either with RA or with DMSO, which is the vehicle for RA, or with various compounds known to induce terminal differentiation of preadipose cell lines. EBs were then maintained in differentiation medium and the percentage of EB outgrowths showing adipocyte colonies was determined 22 days after EB formation. As shown in Fig. 3A, no treatment or treatment for 3 days with DMSO or with adipogenic hormones such as insulin and triiodothyronine (T_3) led to adipogenesis at a low level (5-10%). Similar results were obtained after treatment with the thiazolidinedione BRL49653 or the fatty

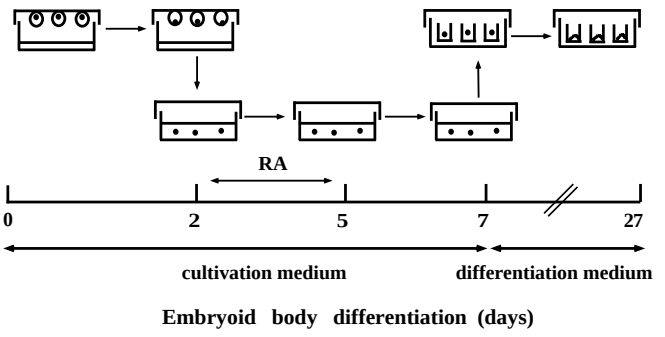

Fig. 1. Schematic presentation of the experimental protocol used for ES differentiation into adipocytes.

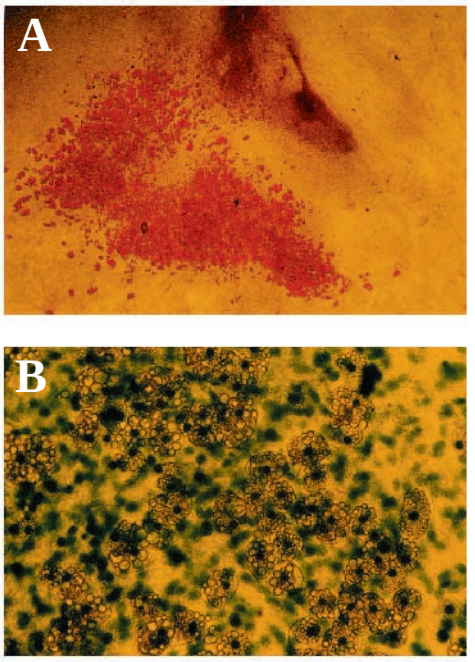

Fig. 2. Photomicrographic record of EB-derived adipocytes. Zin 40 ES cell-derived EBs were allowed to undergo adipocyte differentiation in vitro as described in Materials and Methods. (A) Outgrowths were fixed in 0.2% glutaraldehyde and stained with Oil Red O for fat droplets (Green and Kehinde, 1974). Adipocytes (stained in red) which developed out of the dense center of an EB (stained in brown) are shown (×40). (B) Outgrowths were stained for β-galactosidase activity (Beddington et al., 1989). Adipocytes filled with lipid droplets and with nuclear expression of β-galactosidase are shown (×100).

acid 2-bromopalmitate which are activators of peroxisome proliferator-activated receptors (PPARs), previously described as triggers of adipocyte differentiation (Amri et al., 1995; Brun et al., 1996). In contrast, 60% of EBs previously exposed to RA formed adipocyte colonies. Comparable frequencies of adipogenesis were observed with three different feeder-independent ES cell lines. Treatment before the 2nd day was cytotoxic in our conditions and EBs did not give rise to adipocytes when treated after the 5th day (not shown). The absence either of T_3 or of insulin in the differentiation medium reduced the yield of adipogenesis to only 10-20% of RA-treated EBs. The maximal response was obtained in the presence of both hormones. Altogether, these results indicate that two phases can be distinguished in the development of adipogenesis from ES cells: the first phase, between the 2nd and the 5th day, corresponds to a permissive period for the commitment of ES cells which is influenced by RA. Activators of PPARs or addition of adipogenic hormones at that stage were not sufficient to induce adipogenesis. The second phase corresponds to the permissive period for terminal differentiation and requires adipogenic hormones as previously shown for established preadipose cells.

RA-treated embryoid body outgrowths expressed specific markers for adipocytes

ES cell-derived adipocytes were characterized in more detail. In situ immunostaining analysis revealed that EB outgrowths expressed glycerol-phosphate-dehydrogenase (GPDH) and adipocyte lipid binding protein (ALBP) (not shown) two proteins preferentially expressed in terminally differentiated adipocytes in vitro and in vivo (Dani et al., 1989). The lipogenic and lipolytic activities of ES cell-derived adipocytes were then tested. GPDH is a lipogenic enzyme required for the formation of the glycerol backbone which is present in triglyceride-containing cells in developing mouse adipose tissue (Cook and Kozak, 1982). As shown in Fig. 4A, DMSO-treated EB outgrowths expressed a low level of GPDH activity compared to RA-treated EB outgrowths. The ability to hydrolyse stored triglycerides in response to β-adrenergic agonists is the second important characteristic of mature adipocytes. The lipolytic activity of adipocyte-containing outgrowths is shown in Fig. 4B. As shown, addition of isoproterenol, a β-adrenergic agonist, led to a potent release of glycerol.

Expression of specific genes for adipocytes, such as *adipsin, adipocyte lipid binding protein (ALBP)* (Spiegelman et al., 1983), and *peroxisome proliferative-activated receptor (PPAR)*

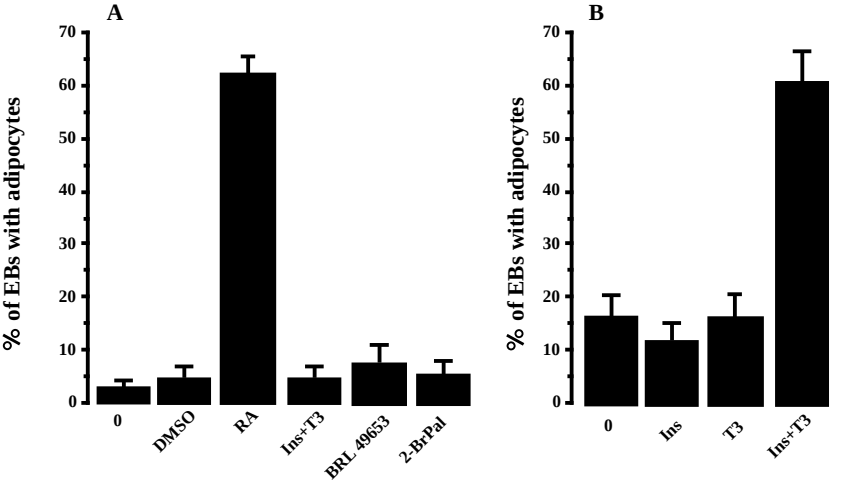

Fig. 3. (A) Influence of RA-treatment on the adipocyte differentiation of ES cells. EBs were treated from 2nd-5th day with indicated compounds: 0.1% DMSO (number of EBs checked n=154); RA (10^{-8} M, n=159); insulin and T_3 (85 nM and 2 nM, respectively, n=140); BRL49653 (10 μM, n=95); 2-bromopalmitate (5 μM, n=90). The percentage of EB showing adipocyte colonies after 15 days in differentiation medium is shown (abscissa). Data were calculated from the differentiation of three different ES cell lines and are the means ± s.e. of three independent experiments. (B) Influence of adipogenic hormones on the adipocyte differentiation of RA-treated EBs. EBs were treated from 2nd-5th day with 10^{-8} M RA and maintained from 7th-22nd day in the absence (0, n=57) or the presence of indicated hormones: insulin (85 nM, n=43); T_3 (2 nM, n=42); insulin and T_3 (85 nM and 2 nM, respectively, n=54). The percentage of EB showing adipocyte colonies at day 22 is shown (abscissa). Data were calculated from the differentiation of three ES cell lines and are the means ± s.e.m of three independent experiments.

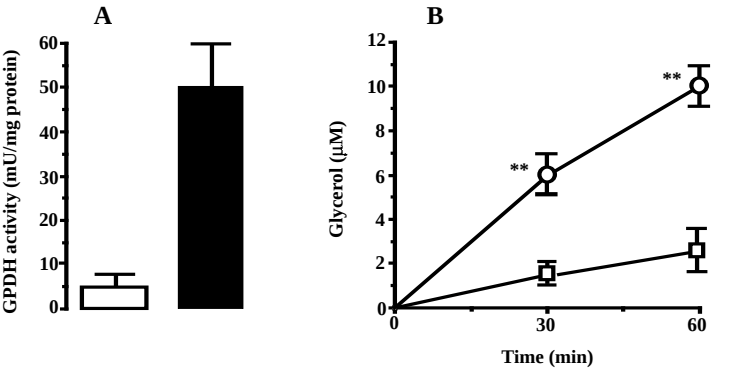

Fig. 4. (A) GPDH activity in RA-treated EB outgrowths. EBs were treated with 0.1% DMSO (white bar) or with 10^{-6} M RA (black bar) from 2nd-5th day and maintained for 12 days in differentiation medium. GPDH activity was determined from a single EB outgrowth. Results are the means ± s.e.m of three independent experiments. (B) Isoproterenol-stimulated glycerol release from an adipocyte-containing EB outgrowth. A single RA-treated EB outgrowth containing (○) or not (□) adipocytes were exposed for the indicated time to 1 μM isoproterenol. Glycerol released into the medium was measured as previously described (Darimont et al., 1994). Results are the means ± s.e.m of four independent experiments. Values significantly different from control (RA-treated EBs outgrowths which did not contain microscopically detectable adipocytes) are indicated by ** (P<0.01).

γ (Tontonoz et al. 1994a) has been investigated by northern hybridisation analysis. These adipogenic markers are undetectable or expressed at low level in pluripotent ES cells and in DMSO-treated cultures. Their expression is induced at a high level in outgrowths from RA-treated EBs (Fig. 5). Expression of various markers of adipogenesis as well as the ability to store and to hydrolyze triglycerides indicate that the ES cell-derived outgrowths contained fully differentiated adipose cells.

In contrast to the induction of adipogenesis, addition of RA decreased formation of beating cardiomyocytes (see Fig. 7). This result is in agreement with a previous publication of Wobus and colleagues who reported that RA, used in a similar experimental procedure to that described in this paper, inhibits cardiogenesis from ES cells (Wobus et al., 1994). In order to know whether the conditions that we used led to generalised changes in the developmental programme of ES cells, expression of *A2COL6* gene was investigated. *A2COL6* gene has been shown to be expressed transiently during differentiation of myoblasts and adipoblasts (Ibrahimi et al., 1993) and chondroblasts (Quarto et al., 1993). More recently, it has been shown that the A2COL6 gene is mainly expressed by mesenchymal cells (Marvulli et al., 1996). As shown in Fig. 5, the RA treatment did not modify the level of A2COL6 mRNA (see also Fig. 8).

Treatment of EBs with RA during defined intervals of time or at different concentrations

In order to determine the period of RA treatment required for induction of adipocyte differentiation, EBs were treated with RA (10^{-8} M) for defined time intervals between the 2nd and the 5th day. Adipogenesis was scored at day 22 by comparing the level of the mRNA encoding for the adipocyte-specific protein ALBP. Fig. 6 shows that expression of the *ALBP* gene was induced by a two-day exposure and the highest level of expression was obtained when RA was maintained for 3 days. The expression of the *ALBP* gene was paralleled by the development of adipocyte colonies scored by microscopic examination (not shown).

The effect of treatment of EBs between the 2nd and the 5th

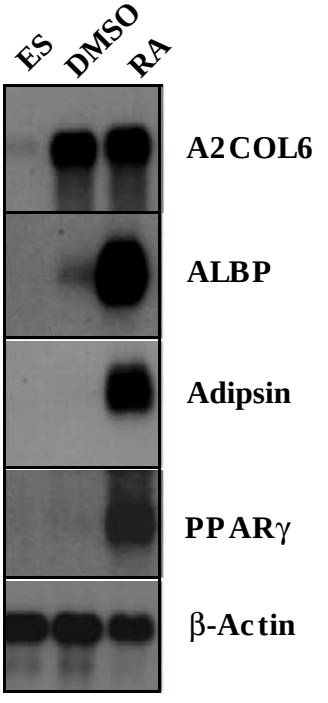

Fig. 5. RA-treated EB outgrowths express adipocyte specific markers. RNA was prepared from undifferentiated ZIN40 ES cells or from 27-day-old EBs treated from 2nd-5th day with 0.1% DMSO or with 10^{-8} M RA. Poly(A)$^+$ RNA (3 μg) samples were fractionated by electrophoresis, transferred to membrane and the same blot was successively hybridized with indicated probes. An equivalent amount of intact poly(A)$^+$ RNA was run in each condition as indicated by hybridization to the β-actin probe.

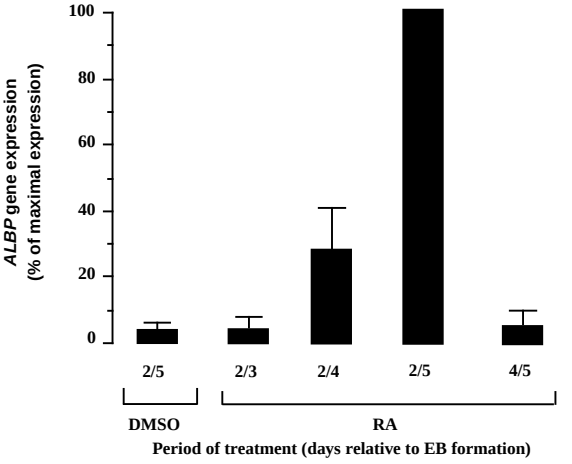

Fig. 6. Treatment of EBs with RA during defined intervals of time. ZIN40 EBs were treated with RA 10^{-8} M for indicated intervals of time and expression of ALBP mRNA was determined in 22 day-old outgrowths. RNAs were analyzed by northern blotting. Quantification of the hybridization signal was performed as described in Materials and Methods and standardized to β-actin mRNA signal. Results are the means of two independent experiments.

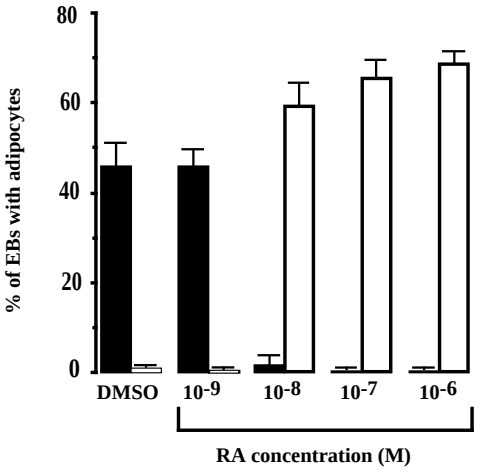

Fig. 7. Treatment of EBs with different concentrations of RA. Two-day-old outgrowths from ZIN40 EBs were treated for 3 days with an increasing concentration of RA. The percentage of EBs showing beating cardiomyocytes (back bars) or adipocyte colonies (white bars) was determined after 5 days or 13 days in differentiation medium, respectively. Data are the means of two independent experiments. 0.1% DMSO, number of EBs n=166;10^{-9} M RA, n=202; 10^{-8} M RA, n=136; 10^{-7} M RA, n=175; 10^{-6} M RA, n=163.

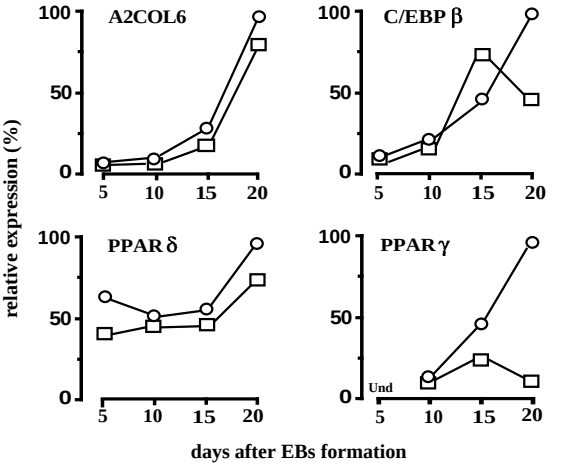

Fig. 8. Kinetics of gene expression in developing EBs. ZIN40 EBs were treated from 2nd-5th with 10^{-8} M RA (○) or with 0.1% DMSO (□). The day of plating in differentiation medium was day 7. Total RNA was prepared at indicated times (days) and 20 μg were subjected to northern analysis. The same blot was hybridized to indicated probes. Quantification of the hybridization signal was performed as described in Materials and Methods and standardized to β-actin mRNA signal. Values are expressed by taking as 100% the maximal signal obtained for each probe. Und, undetectable.

day with different concentrations of RA was then determined. The percentage of EB outgrowths containing adipocyte colonies was determined 20 days after EB formation. As shown in Fig. 7, 10^{-9} M RA did not induce adipogenesis whereas 10^{-8} M gave rise to 54% of EB outgrowths containing adipocytes. Higher RA concentrations (10^{-7} M and 10^{-6} M) led to adipogenesis of ES cells at a similar level. The percentage of EB outgrowths containing beating cardiomyocytes was also evaluated. As previously reported, 10^{-8} M RA and higher concentrations led to a total inhibition of beating cardiomyocytes (Wobus et al., 1994).

Expression of potential adipogenic genes in developing EBs

The kinetics of expression of PPARδ (Amri et al., 1995), (also named FAAR, PPARβ or Nuc-1); PPAR γ (Tontonoz et al., 1994b) and CCAAT/enhancer binding protein (C/EBP) β (Yeh et al., 1995) were investigated by northern hybridisation of RNA prepared from developing outgrowths derived from DMSO- or RA-treated EBs. These nuclear factors regulate genes involved in lipid metabolism and are able to trigger terminal differentiation of preadipocytes into adipocytes (see Discussion). PPARδ and C/EBP β are expressed in several tissues whereas PPAR γ is preferentially expressed in the adipose tissue (Tontonoz et al., 1994b). Expression of the *A2COL6* gene was used as a control for differentiation of mesenchymal cells. As shown in Fig. 8, expression of the *A2COL6* gene increased identically in both cultures. 50% of the maximal *PPAR*δ gene expression was reached in 5-day-old EBs. Subsequently, the expression of the *PPAR*δ gene increased and remained similar in DMSO- and RA-treated developing EB outgrowths. In contrast, *PPAR*γ gene expression was undetectable until day 10 in both conditions. Thereafter, the *PPAR*γ mRNA level remained low in outgrowths from DMSO-treated EBs but increased dramatically in RA-treated cultures coincident with the appearance of mature adipocytes. *C/EBPβ* gene expression was low at day 5 and did not appear to be modulated by RA pre-treatment. The increase in C/EBPβ mRNA in DMSO-pretreated cultures confirms that this nuclear factor is expressed in various differentiating cell types. The relative abundance of these RNAs during adipogenesis of ES cells is shown in Fig. 9. The increase in C/EBPβ mRNA paralleled the increase in PPARγ. This observation is in agreement with the model in which *C/EBPβ* is involved in the induction of *PPAR*γ gene expression in adipose cells (Wu et al., 1995). Expression of the *PPAR*δ gene is early compared to the expression of *PPAR*γ. It has been previously reported that *PPAR*δ gene expression is detected early during embryogenesis and precedes *PPAR*γ (Kliewer et al., 1994). The temporal pattern of expression of these two PPARs during embryogenesis and in developing EBs is conserved. This suggests that ES cell differentiation can mimic closely the development of adipose cells in vivo as previously demonstrated for other programmes of ES cell differentiation (for reviews see Pedersen, 1994; Keller, 1995).

DISCUSSION

We describe in this paper the capacity of ES cells to undergo adipogenesis at a high rate in vitro after treatment of developing ES cell derived-EBs with RA. The ability of RA to induce various differentiation processes has been previously reported for mouse pluripotent P19 embryonal carcinoma cells (McBurney et al., 1982). More recently, it has been shown that, depending on the concentration and the period of treatment, RA was able to induce or to repress differentiation programmes in ES cell cultures. For example, RA promotes the differentiation of ES cells into functional neurons (Bain et al., 1995;

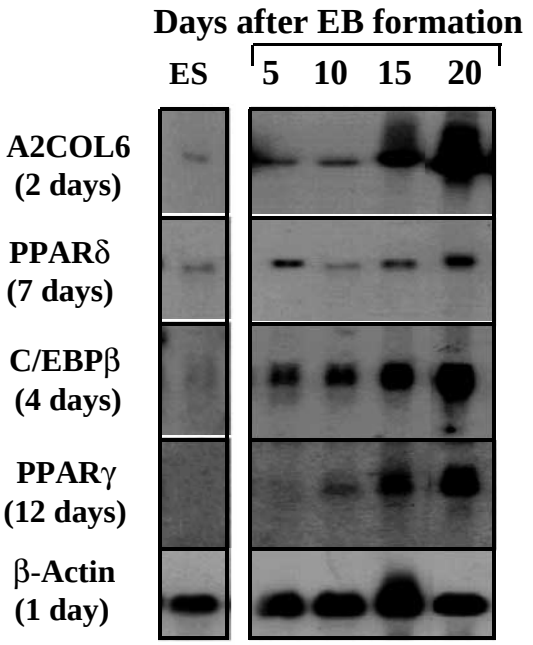

Fig. 9. Expression of adipogenic genes in RA-treated developing EBs. Adipogenesis of ZIN40 ES cells was induced according to the protocol described in Fig. 8 and expression of indicated genes was analyzed by northern blot. Equivalent amounts of intact RNA were run in each lane as indicated by hybridization to β-actin cDNA probe. The time of exposure for each probe is indicated in brackets. ES, pluripotent ES cells maintained in the presence of LIF.

Strübing et al., 1995), and glial cells (Fraichard et al., 1995). In contrast, RA inhibits cardiogenesis from ES cells (Wobus et al., 1994) and expression of mesodermal genes such as Brachyury, Zeta-globin and cardiac-specific isoforms of actin (Bain et al., 1996). To our knowledge this study is the first report that ES cells are able to give rise to mature adipocytes. Adipogenesis from ES cells at a high frequency requires the use of adipogenic hormones involved in terminal differentiation of preadipocytes into adipocytes. The serum we included in the differentiation medium was pre-selected with caution to give maximal terminal differentiation of preadipose cells from mesenchymal clonal lines. However, this parameter did not appear to be crucial as other batches of sera were also effective and the percentage of RA-treated EBs which formed adipocytes ranged from 30% to 60% (data not shown). The essential requirement to commit ES cells into the adipocyte lineage at a high rate was to treat early developing EBs with RA at a concentration higher than 10^{-9} M and for short period of time. During the defined period of time of treatment, between the 2nd and 5th day, EBs consist of undifferentiated embryonic stem cells, cells of the extraembryonic endodermal lineage and cells of embryonic ectodermal lineage which are precursors of definitive ectoderm, endoderm and mesoderm (Robertson, 1987). The narrow window for the effect of RA suggests that it is acting on this latter population.

Present knowledge suggests that adipose cells and skeletal muscle cells are derived from the same mesodermal stem cell precursor (Taylor and Jones, 1979). A switch from cardiogenesis to skeletal myogenesis after RA treatment of ES cell-derived EBs has been reported previously (for discussion see Wobus et al., 1994). Interestingly, a switch from cardiogenesis to adipogenesis was observed during our study (see Fig. 7). RA could induce multipotential mesenchymal stem cells to form which will give rise subsequently to adipose cells in permissive media and/or it may directly induce a master(s) gene(s) for adipogenesis. In vitro differentiation of ES cells could offer a powerful developmental model to analyse molecular events leading to the commitment of the stem cell precursor towards the myogenic or the adipogenic lineage.

The role of RA in early events of the adipocyte development programme can be distinguished from a function described previously in the terminal differentiation of preadipose into adipose cells from established clonal lines. It has been shown that high concentrations of RA (micromolar range) inhibit adipose conversion, whereas lower concentrations of RA (nanomolar range) stimulate adipocyte differentiation. However, in that case RA was only effective in the terminal differentiation process like other adipogenic hormones (Safonova et al., 1994). Our data indicate that transient treatment of EBs with RA from 10^{-8} M to 10^{-6} M induced adipogenesis of ES cells. These results suggest that RA may play a role both in the earliest and in the latest stage of the programme of adipose cell differentiation.

Differentiation of preadipose cells from established clonal lines is accompanied by a rapid emergence of *trans*-acting nuclear factors which play a key role in the regulation of the adipogenic differentiation programme. In addition, it has been clearly shown that ectopic expression of peroxisome proliferator-activated receptor isoforms (PPAR α, γ and δ) in 3T3 fibroblasts induced the expression of adipocyte specific genes (Tontonoz et al., 1994a; Amri et al., 1995). Similar results have been obtained following ectopic expression of the CCAAT/enhancer binding proteins α and β (Freytag et al., 1994; Yeh et al., 1995). However, with the exception of PPARγ, these factors are not specific for the adipocyte lineage since they are expressed in various tissues. Therefore, it seems unlikely that their expression alone can be responsible for adipose-specific commitment. More recently, it has been demonstrated that PPARγ acts as a 'master' regulator that can trigger the entire programme of adipogenesis in fibroblast cells (Brun et al., 1996). The fact that potent activators of PPARs, such as the thiazolidinedione BRL49653 or the fatty acid 2-bromopalmitate, could not commit ES cells into the adipocyte lineage (see Fig. 3) strongly suggests that PPARs do not play a critical role in the first steps of adipose cell development. The combination of genetic manipulations of undifferentiated ES cells, such as gain or loss of function, and in vitro adipocyte differentiation will facilitate elucidation of the role of genes known to be expressed during adipose cell conversion. In addition this culture system should provide a means to identify novel regulatory genes involved in early determinative events in adipogenesis.

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