Retinoic acid stimulates expression of thrombomodulin, a cell surface anticoagulant glycoprotein, on human endothelial cells

Differences between up-regulation of thrombomodulin by retinoic acid and cyclic AMP

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Thrombomodulin (TM) is a surface protein on endothelial cells, and represents one of the most valuable regulatory factors in the anticoagulant system. In this paper, we demonstrate that retinoic acid (RA) causes an increase in TM antigen on human umbilical vein endothelial cells (HUVECs) *in vitro*. The effect of RA on the surface TM level of HUVECs was dose-dependent in the range from 0.01 to 10 μ M-RA. Antigen levels began to increase 3 h after addition of 10 μ M-RA, and plateaued at a maximum level of approx. 2.5 times that of the untreated control at 24 h. TM levels remained at a maximum for a further 12 h, and then gradually decreased. The effects of RA on cell surface TM activity and antigen levels were parallel in all experiments. TM expression was also increased by treatment with 10 μ M-retinal or 10 μ M-retinol for 24 h, though the increases were approx. 70 % and 30 % respectively of that produced by 10 μ M-RA. Pretreatment of HUVECs with cycloheximide inhibited the effect of RA. When HUVECs were incubated with both 10 μ M-RA and 5 mM-8-bromo cyclic AMP (or 1 mM-3-isobutyl-1-methylxanthine, a phosphodiesterase inhibitor), the increase in TM antigen was greater than that observed with either compound alone. Northern blot analysis showed that treatment of HUVECs with 8-bromo cyclic AMP, RA or RA plus 8-bromo cyclic AMP increased TM mRNA levels by 2.2-, 4.5- and 5.5-fold respectively compared with the untreated control. Furthermore, no significant difference in cellular cyclic AMP levels was observed between RA-treated and control cells. These results indicate that the expression of TM is not only controlled by the intracellular cyclic AMP level but is also affected by RA, and suggest that RA-induced up-regulation of TM on HUVECs is independent of cyclic AMP regulation.

INTRODUCTION

Thrombomodulin (TM) is an endothelial membrane surface glycoprotein with an anticoagulant function, in that it accelerates thrombin-catalysed activation of protein C [1–4]. Recent studies *in vitro* have shown that the expression of TM on cell surfaces is down-regulated through the actions of interleukin-1 β and tumour necrosis factor- α , which are released from activated monocytes and macrophages [5–10]. This may be important in pathological conditions such as disseminated intravascular coagulation, since both cytokines also increase the production of tissue factor, the initiation factor of the extrinsic clotting pathway, on the surface of endothelial cells [6–9,11,12]. Since TM functions as an antithrombotic agent by altering thrombin specificity and supporting protein C activation, control of TM expression on endothelial cells is necessary for maintenance of a normal coagulant balance.

Retinoic acid (RA, vitamin A acid) is widely accepted as a regulatory compound involved in cell proliferation, differentiation and pattern formation during development [13–21]. On treatment of mouse teratocarcinoma F9 cells with RA, the embryonal carcinoma stem cells were changed into primitive endoderm cells, and subsequently differentiated into parietal endoderm when the cultures were exposed to dibutyryl cyclic AMP (cAMP) [13,22]. Recently, fetomodulin (FM), a surface marker protein of mouse parietal endoderm, was identified as mouse TM by gene cloning and functional assay techniques [23]. Although FM is present neither in embryonal carcinoma stem

cells nor in primitive endoderm cells, it first appears in parietal endoderm cells after treatment with dibutyryl cAMP [24]. Imada *et al.* [23] also found that FM was localized not only in the vasculature, but also in tissues that were not directly exposed to body fluids in the mouse embryo, and they suggested that it might be a multifunctional protein with a unique role in embryonic development. Thus studies on changes in TM antigen levels and the regulatory mechanisms of TM expression under various conditions become extremely important.

It is known that TM is up-regulated by treatment with analogues of cAMP in human umbilical vein endothelial cells (HUVECs) [25,26] and human megakaryoblastic leukaemia cells [27]. However, no data have been reported concerning the effect of RA in the presence or absence of cAMP on protein synthesis in HUVECs. Here we investigate whether or not RA can modify the expression of TM on the surface of HUVECs.

EXPERIMENTAL

Materials

All-*trans*-RA, retinal (*trans*), retinol (*trans*), dibutyryl cAMP, 8-bromo cAMP, 3-isobutyl-1-methylxanthine (IBMX), heparin (pig intestinal mucosa), BSA, *o*-phenylenediamine, cycloheximide and actinomycin D were obtained from Sigma, St. Louis, MO, U.S.A. Boc-Leu-Ser-Thr-Arg-MCA and 7-amino-4-methylcoumarin were purchased from the Peptide Institute, Osaka, Japan. Dulbecco's minimal essential medium (DMEM) and fetal calf serum (FCS) were purchased from Flow Laboratories,

Abbreviations used: TM, thrombomodulin; FM, fetomodulin; RA, retinoic acid; cAMP, cyclic AMP; HUVECs, human umbilical vein endothelial cells; IBMX, 3-isobutyl-1-methylxanthine; DMEM, Dulbecco's minimal essential medium; FCS, fetal calf serum; ECGS, endothelial cell growth supplement; DMSO, dimethyl sulphoxide; HRP, horseradish peroxidase; e.i.a., enzyme immunoassay; Boc, t-butoxycarbonyl; MCA, 4-methylcoumarin-7-amide.

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Fig. 1. Effect of RA on surface and total TM antigen levels and surface TM cofactor activity in HUVECs

HUVEC cultures (96-well dishes) were treated with $10 \mu\text{M}$ -RA for various times and the TM antigen level was measured on the cell surface (a) and after solubilization of the cells (b) by e.i.a. using HRP-labelled TM monoclonal antibodies (TMmAbs 2/11 and 2/11/20 respectively). The surface cofactor activity of TM (c) was determined by measuring the generation of activated protein C using a synthetic substrate as described in the Experimental section. RA (■) was dissolved in DMSO (0.1% final concentration), and the controls (□) included 0.1% DMSO instead of RA. Specific binding was calculated by subtracting the amount of non-specific binding from total binding as described in the Experimental section. Results in (a) are expressed as percentages of the initial level in the control, and all data are means $\pm$ S.D. from four independent experiments and cell strains.

Irvine, Scotland, U.K., and Hyclone Laboratories, Logan, UT, U.S.A., respectively. Collagen-coated dishes (60 mm diameter) and 24- or 96-well tissue culture plates were purchased from Corning-Iwaki Glass, Tokyo, Japan, and Falcon, Becton Dickinson and Company, Lincoln Park, NJ, U.S.A., respectively. The tissue culture plates (24 and 96 wells) were coated with gelatin

before use. Endothelial cell growth supplement (ECGS) and trypsin were obtained from Collaborative Research, Waltham, MA, U.S.A., and DIFCO Laboratories, Detroit, MI, U.S.A., respectively. The cAMP r.i.a. assay kit was obtained from Yamasa Co., Chiba, Japan. Nylon cellulose membrane (Hybond-N) was purchased from Amersham Japan. [^{32}P]dCTP was purchased from NEN, and the nick-translation kit was from Nippon Gene, Tokyo, Japan. Restriction enzymes and a 7-DEAZA sequencing kit were obtained from Takara Shuzo Ltd., Osaka, Japan. Other reagents were purchased from Wako Pure Chemicals, Osaka, Japan. Thrombin was donated by the Green Cross, Osaka, Japan, and was further purified by benzamidine-Sepharose column chromatography. Protein C was purified from human plasma by BaSO_4 precipitation, $(\text{NH}_4)_2\text{SO}_4$ fractionation and sequential column chromatography on DEAE-Sephacel, heparin-agarose, and finally on Mono Q f.p.l.c. according to the method of Suzuki *et al.* [28]. Mouse monoclonal anti-(human TM) IgGs (TMmAbs 2, 11 and 20) were prepared and labelled with horseradish peroxidase (HRP) as reported previously [29]. TMmAb20 recognizes a thrombin-binding site of TM and inhibits the cofactor activity of TM in protein C activation by the TM-thrombin complex, whereas TMmAbs 2 and 11 neither recognize the binding site nor inhibit the activity. These TMmAbs do not compete with each other for binding to TM [29].

Cell culture

Endothelial cells were isolated from human umbilical cord veins according to the method of Jaffe *et al.* [30], and were cultured in 60 mm-diam. collagen-coated dishes containing culture medium A, which consisted of DMEM supplemented with 20% (v/v) FCS, $20 \mu\text{g}$ of ECGS/ml, $100 \mu\text{g}$ of heparin/ml and antibiotics ($50 \mu\text{g}$ of streptomycin/ml and $50 \mu\text{g}$ of penicillin/ml). Confluent primary cultures were passed and cultured under the same conditions to confluency and then seeded in gelatin-coated 24- or 96-well plates. These cells were grown to confluency under 5% CO_2 and were used for the present experiments. FCS did not affect the RA-dependent expression of TM in HUVECs.

Cell treatment

Culture medium A was removed from the cell cultures and $200 \mu\text{l}$ of medium A containing each retinoid and/or the agents shown in the Figures was added. Retinoids were dissolved in 0.1% (v/v) dimethyl sulphoxide (DMSO), and 0.1% DMSO was used as the control. The treated cells were incubated at 37°C in a CO_2 incubator and washed once with medium B [0.2% (w/v) BSA and 20 mM-Hepes, pH 7.4] before measurement of TM antigen levels.

Determination of TM antigen levels

The cell surface TM antigen levels were measured by the method described previously [25]. In short, the cultures treated with various agents were washed once with medium B, and then HRP-labelled monoclonal antibodies against TM (equal amounts of TMmAbs 2 and 11) in medium A were added. After 10 min, the dishes were washed three times with medium A and the products of the H_2O_2 -peroxidase-coupled reaction were measured at 492 nm by a Bio-Rad enzyme immunoassay (e.i.a.) reader [29]. Specific binding was calculated from the total IgG bound by subtracting the amount of non-specific binding measured after addition of a 100-fold excess of non-labelled TMmAbs. The data were expressed as percentage binding relative to control values [29]. Total TM antigen in HUVECs was measured as described [29] in cells extracted with 50 mM-Tris/HCl, pH 7.5, containing 0.15 M-NaCl, 0.5% (w/v) Triton X-100 and 1 mM-benzamidine hydrochloride for 30 min at 4°C . The data were expressed as an absolute amount of TM.

Fig. 2. Effect of RA concentration on surface TM antigen levels and cofactor activity in HUVECs

HUVEC cultures were treated with various concentrations of RA for 24 h, and the surface TM antigen level (a) and surface TM activity (b) were measured as described in the Experimental section. The activated protein C activity was 0.95 ± 0.13 nmol/min per well in the control. Results are expressed as percentages of the control value, which was measured with 0.1% DMSO. Data are the means $\pm$ s.d. from four independent experiments and cell strains.

Determination of surface TM activity

The cofactor activity of cell surface TM was determined by measuring the amidolytic activity of activated protein C, which was generated by the cell surface TM in the presence of 50 μ g of human protein C/ml, 2 NIH units of thrombin, 1 mM- CaCl_2 and 5 mg of BSA/ml in 20 mM-Tris/HCl, pH 7.4, containing 0.1 M-NaCl [25]. After incubation for 90 min at 37 $^\circ\text{C}$ in a CO_2 incubator, thrombin activity was terminated by addition of a mixture of antithrombin III (final concentration 2 units/ml) and heparin (final concentration 8 units/ml). A mixture of the conditioning medium and Boc-Leu-Ser-Thr-Arg-MCA, a synthetic substrate of activated protein C, was incubated in a test tube for 10 min at 37 $^\circ\text{C}$ and the reaction was terminated by adding acetic acid to a final concentration of 10% (v/v). The 7-amino-4-methylcoumarin liberated was then measured by using a spectrofluorimeter with excitation at 380 nm and emission at 460 nm.

Northern blot analysis

Total RNA was prepared from HUVECs (cultured in 5–10 $\times$ 60 mm-diam. dishes) by the guanidinium thiocyanate method [31]. After agarose gel electrophoresis, the RNA was transferred to a nylon filter and hybridized with a labelled cDNA probe. The TM cDNA for the probe was obtained from 8×10^5 clones by screening of a human placental cDNA library in λ gt11 with TM polyclonal and monoclonal antibodies. A TM cDNA probe (415 bp) for the hybridization, corresponding to nucleotides 803–1217 of human TM cDNA, was obtained by digestion of the cDNA with both *Tth*111I and *Nhe*I. The nucleotide sequence determination was carried out by a modified dideoxy chain termination method using a 7-DEAZA sequencing kit, and the sequence was identical to that of the nucleotides in human TM cDNA [32,33]. The probe corresponding to the epidermal growth factor-like domain (58% of the total bases for the domain) was labelled with [^{32}P]dCTP (3000 Ci/mmol) using a nick-translation kit. The filter was rehybridized with a [^{32}P]cDNA probe of human β -actin after washing with water [26]. Auto-

radiographs were densitometrically analysed with a Shimadzu flying-spot scanner (CS-9000) (Kyoto, Japan).

RESULTS

The effects of RA on surface and total TM antigen levels in HUVECs and on surface TM cofactor activity were investigated (Fig. 1). At 3 h after addition of 10 μM -RA the relative TM antigen level on the cell surface had increased, reaching a maximum level by 24 h (Fig. 1a). The approx. 2.5-fold increase in TM antigen levels was maintained for a further 12 h, after which it gradually decreased. The time course of the change in total TM antigen levels after treatment with RA (Fig. 1b) was similar to that observed on the cell surface. An increase in TM cofactor activity on the cell surface induced by RA paralleled that observed for total and surface TM antigen levels (Fig. 1c). The effects of increasing concentrations of RA on cell surface TM antigen and cofactor activity were then examined after incubation for 24 h (Fig. 2). Changes in TM expression depended entirely upon the amount of RA, and a significant increase in TM levels was observed at 10^{-8} M-RA; 10^{-5} M-RA was required to reach the maximum TM level under the conditions employed. The change in surface cofactor activity was again synchronous with the change in the surface TM antigen level. These results indicate that RA treatment increases the TM level in HUVECs, and that the newly-formed TM was as functional as the existing TM.

The expression of surface TM antigen after treatment of HUVECs with vitamin A and its metabolites was compared (Fig. 3). Judging by the profiles of the time course of TM expression, retinol (vitamin A) and retinal (aldehyde form of vitamin A) had the same effect as RA (acid form of vitamin A), although the potencies were less than that of RA. At 24 h after addition of 10 μM -retinal or 10 μM -retinol, the maximum levels of TM expression were 72% and 29% respectively of that observed with 10 μM -RA. When the retinal and retinol concentrations were 50 μM , TM expression increased to 92% and 51% respectively of that with 50 μM -RA (results not shown).

Fig. 3. Effects of retinol and retinal on the expression of surface TM antigen in HUVECs

Cultures were treated with 10 μ M-retinol (◆), -retinal (□) or -RA (■) for 24 h and the cell surface TM antigen levels were measured as described in the Experimental section. Results are expressed as percentages of the control value, which was measured with 0.1% DMSO. Data are the means of results from three independent experiments.

It is known that cAMP modulates TM expression in HUVECs, and we reported that treatment of HUVECs with dibutyl cAMP, 8-bromo cAMP or IBMX, a phosphodiesterase inhibitor, resulted in increases in total and cell surface TM antigen levels [25]. We investigated whether the simultaneous treatment of HUVECs with 8-bromo cAMP (or IBMX) and RA resulted in an additional increase in surface TM antigen level (Fig. 4). Treatment of HUVECs with 5 mM-8-bromo cAMP, 1 mM-IBMX or 10 μ M-RA resulted in increases of 175%, 185% and 250% respectively in TM levels after incubation for 24 h. Additional increases in TM levels (400% and 420% respectively) were observed in the cells treated for 36 h with 5 mM-8-bromo cAMP

plus 10 μ M-RA or 1 mM-IBMX plus 10 μ M-RA. When HUVECs were treated with RA (10 μ M) for 12 h followed by 8-bromo cAMP (5 mM), or with 8-bromo cAMP (5 mM) for 12 h followed by RA (10 μ M), TM expression eventually increased to the level observed with simultaneous treatment of the cells (Fig. 4a). Similar results were obtained when 1 mM-IBMX was added instead of the 8-bromo cAMP (Fig. 4b).

The ability of RA or RA plus 8-bromo cAMP to stimulate TM expression in HUVECs was abolished by pre-treatment of cells with 10 μ g of cycloheximide/ml for 1 h. This treatment had no effect on cell viability, indicating that RA and/or cAMP were affecting protein biosynthesis rather than suppressing degradation (results not shown). Northern blotting was performed to compare the TM mRNA levels after treatment of HUVECs with RA, RA plus 8-bromo cAMP and RA plus IBMX (Fig. 5). Total RNA was extracted from HUVECs after 24 h of incubation with 10 μ M-RA and/or the other agents, and was electrophoresed, transferred, and then hybridized with a 32 P-labelled TM cDNA probe. RA treatment induced an increase in TM mRNA levels in HUVECs, and this increase was enhanced by simultaneous treatment with 8-bromo cAMP or IBMX. In the densitometric analysis of the autoradiogram, the bands corresponding to TM mRNAs from cells treated with 8-bromo cAMP (Fig. 5, lane 2), IBMX (lane 3), RA (lane 4), RA plus 8-bromo cAMP (lane 5) and RA plus IBMX (lane 6) were increased 2.2, 2.8, 4.5, 5.5 and 6.3 times respectively compared with the control (lane 1), after normalization of TM mRNA levels to β -actin mRNA levels. The effect of actinomycin D on TM mRNA levels in HUVECs treated with RA was examined by dot-blot hybridization (Fig. 6). The RA-dependent increase in TM mRNA levels was lessened by simultaneous treatment of the cells with actinomycin D. These results indicate that the enhanced expression of TM antigen in RA-treated HUVECs results from an increase in TM transcription.

DISCUSSION

It has been reported that retinol treatment (10 μ M for 2 days) produces morphological changes in bovine carotid artery endothelial cells from a cobblestone-like arrangement to a spindle-

Fig. 4. Elevation of cell surface TM antigen levels after treatment of HUVECs with RA plus 8-bromo cAMP or RA plus IBMX

(a) RA (10 μ M) and 8-bromo cAMP (5 mM) were added separately or together into cell-conditioned medium. (b) RA (10 μ M) and IBMX (1 mM) were added separately or together into cell-conditioned medium. At the indicated times, cell surface TM antigen was measured as described in the Experimental section. The arrows at 12 h indicate the cultures to which 10 μ M-RA, 5 mM-8-bromo cAMP (8Br) or 1 mM-IBMX was added, and the subsequent broken lines represent the changes in TM levels after the addition of each agent. Results are expressed as percentages of the initial control level, and data are the means of results from three independent experiments. ■, RA; □, 8-bromo cAMP (a) or IBMX (b); ◇, RA plus 8-bromo cAMP (a) or RA plus IBMX (b).

Fig. 5. TM mRNA levels in HUVECs after treatment with 8-bromo cAMP, IBMX and RA

(a) Total RNA was prepared from HUVECs treated for 24 h with vehicle only (control, lane 1), 5 mM-8-bromo cAMP (lane 2), 1 mM-IBMX (lane 3), or 10 μ M-RA (lane 4). Cells were also treated with 10 μ M-RA in the presence of 5 mM-8-bromo cAMP (lane 5) or 1 mM-IBMX (lane 6). After separation by agarose gel electrophoresis and blotting on to a nylon membrane, the RNA was hybridized with a 32 P-labelled TM cDNA probe as described in the Experimental section. The positions of size markers are indicated. (b) The autoradiogram in (a) was analysed by densitometry, and the data are expressed as percentages of the control after normalization to β -actin mRNA levels. Abbreviation: 8Br, 8-bromo cAMP.

Fig. 6. Effect of actinomycin D on the RA-dependent increase in TM mRNA

HUVECs were treated with 10 μ M-RA in the presence or absence of 1 μ g of actinomycin D (AD)/ml for 6 h, and total RNA was isolated as described in the Experimental section. Total RNA (10 or 20 μ g) was blotted on to a nylon membrane and hybridized with the 32 P-labelled TM cDNA probe.

like shape [34]. We also found similar morphological alterations in HUVECs exposed to RA (Fig. 7). Although these shape changes resemble those observed in HUVECs treated with interleukin-1 β or tumour necrosis factor- α , TM expression declines with cytokine treatment, in contrast with the RA-induced stimulation (results not shown) [5,6,9,35,36]. Pober *et al.* [35,36] reported that the shape change caused by these cytokines might be a function of an altered progression through the cell cycle. The shape change in endothelial cells caused by RA might also be induced by a change in the cell cycle, although the action of RA is probably different from that of the cytokines.

In the present paper we have demonstrated that the vitamin A derivatives retinal, retinol and RA induced expression of TM antigen in HUVECs in a time- and dose-dependent manner. Increased TM expression occurred at physiological levels (10^{-8} M-RA), since the concentrations of RA in human serum [37,38] and rat tissues [38] are within the range $(1.2-1.6) \times 10^{-8}$ M and $(0.5-1.0) \times 10^{-7}$ M respectively, suggesting that RA in circulating plasma can regulate TM expression in endothelial cells. The up-regulation of TM also occurred when HUVECs were treated with retinal or retinol. This stimulatory action of the retinoids is likely to be an effect of RA rather than of the precursor, retinal or retinol, because RA is at least an order of magnitude more potent than retinol in various cellular actions [39]. However, we cannot exclude the possibility that retinal or retinol alone are capable of up-regulating TM in HUVECs, since the effects of both agents on the expression were the same as that of RA with respect to the profiles of the time course changes (Fig. 3). Nara *et al.* [34] showed that treatment of bovine endothelial cells with 10 μ M-retinol stimulated production of transglutaminase after 6 h and that the effect was greater than that in cells treated with the same concentration of RA. Thus it appears that the mechanism of RA-dependent induction of TM in HUVECs is different from that of retinol-dependent induction of transglutaminase in bovine endothelial cells.

It has been reported that an increase in the level of cAMP in HUVECs induces TM up-regulation [25,26], and that dibutyl cAMP increases the TM mRNA levels in these cells [26]. We confirmed that TM mRNA levels also increase after treatment with 8-bromo cAMP or IBMX, a phosphodiesterase inhibitor, which are known to up-regulate TM antigen levels on HUVECs. The present paper further indicated that RA enhanced TM biosynthesis via an elevation of TM mRNA levels in HUVECs. The maximal up-regulation of TM biosynthesis by RA was enhanced further by elevation of the mRNA levels by pre-, post- or simultaneous treatment of HUVECs with 8-bromo cAMP or IBMX. This may suggest the existence of different regulatory

Fig. 7. RA-induced morphological changes in cultured endothelial cells

Third-pass HUVECs were treated with 10 μ M-RA for 24 h. (a) 0.1% DMSO-treated control cells; (b) RA-treated cells.

mechanisms for cAMP- and RA-induced expression of TM on HUVECs. This suggestion was supported by the observation that there was no significant difference in cAMP content between 10 μ M-RA-treated cells (12 h incubation) (0.57 ± 0.16 pmol/ 10^5 cells) and the untreated controls (0.49 ± 0.13 pmol/ 10^5 cells). It has been reported that the activator protein-2 domain is one candidate for a cAMP-responsive element in the TM gene by analysis of consensus nucleotide sequences [33,40,41]. However, the typical responsive elements for RA receptor proteins [42–47] which are involved in the initiation of gene transcription, such as a perfect direct repeat of the motif *GTTCAC* in the RA receptor β gene [45] or a consensus activator protein-1 site *TGACTCA* [47], are absent in the 5'-flanking region of the TM gene. Since nucleotide sequences such as *GTGCAC* and *GATCAC*, at nucleotides –586 and –616, respectively, exist upstream of the TATA box in the human TM gene, it is possible that an unidentified responsive element may function for the RA receptor protein. Alternatively, RA may cause production of a specific regulatory protein which directly or indirectly modulates transcription of the TM gene. Chiocca *et al.* [48] reported that RA caused an accumulation of tissue transglutaminase mRNA in macrophages in a rapid and direct manner, and that this effect was not mediated by an increase in the stability of the mRNA but by an increase in the relative transcription rate of the gene. We confirmed that the increase in TM mRNA levels by RA is also not due to changes in TM mRNA stability, since the increase was prevented by pre-treatment of HUVECs with actinomycin D (Fig. 6). Thus an increase in TM expression caused by RA is due to an enhanced rate of gene transcription.

It is known that F9 cells change to primitive endoderm after RA treatment and subsequently become parietal endoderm cell after cAMP treatment [13,22]. FM, which is identical to TM, was induced markedly by treatment of primitive endoderm cells with cAMP [24], although the protein synthesis was seen neither in the embryonal carcinoma stem cells treated with cAMP nor in the primitive cells treated with RA. On the other hand, up-regulation of TM occurs in HUVECs treated with either cAMP or RA. It will be of interest to clarify why FM/TM expression is affected differently in the two cell types (HUVECs and F9 cells) by treatment with cAMP and RA. The distinct responses may be associated with differences in cell differentiation. Further studies on the differences between RA-dependent responses in F9 cells and HUVECs may contribute towards understanding other potential functions of TM, in addition to elucidating the mechanism of RA action in cell differentiation.

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