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PLASMINOGEN ACTIVATOR ACTIVITY OF NORMAL AND RETINOIC ACID-TREATED POST-IMPLANTATION EMBRYOS

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Abstract—Urokinase-type plasminogen activator (uPA) has been implicated in cellular migration accompanying different biological phenomena including organogenesis. An increase in uPA activity was observed in mouse post-implantation embryos during the early organogenesis period. Since we have previously shown that all-trans retinoic acid (RA) prevented the induction of uPA in mouse peritoneal macrophages, we have now assessed whether teratogenic doses of this agent could also interfere with uPA activity in mouse embryo *in vitro* and *in vivo*.

Post-implantation embryos (8.5 days) were incubated for up to 24 hr with micromolar concentration of RA resulting in the occurrence of malformations. No significant difference in uPA activity was found between control and treated embryos. Likewise, uPA activity was not altered in embryos explanted on day 9.5 from dams treated 24 hr before with a teratogenic dose of RA. This study indicates that the teratogenic activity of RA is not caused by an inhibition of the induction of uPA in embryos.

Different biological processes involving cell movements have been associated with an important proteolytic activity focally degrading the extracellular matrix and breaking the intercellular bridges. In particular, tissue (re)modeling and migration of adult and embryonic cell types have been closely linked to increased plasminogen activator (PA; EC 3.4.21.31) activity [1, 2]. For example, PA has been implicated in migration of neuronal [3], epidermal [4], inflammatory [1, 2], neoplastic [5, 6] and endothelial cells [7]. The role of PA in the invasive process implicated in trophoblast implantation in the endometrium has been stressed. It has been shown by Strickland *et al.* [8] that mouse trophoblast cells produce PA when they invade the uterine decidua during implantation. The enzyme production by these cells is closely correlated with the invasive period of trophoblast cells and suggests that PA is involved in embryo implantation [8, 9].

PA has also been identified in the post-implantation mouse embryo [8–11]. PA may thus play an important role in development, tissue modeling and migratory processes accompanying embryogenesis.

Retinoids are a group of chemicals which influence epithelial and mesenchymal tissue differentiation [12]. Retinoic acid (RA) is a metabolite of vitamin A, displaying a pronounced teratogenic activity [13, 14]. The exact mechanism of this teratogenic activity is still obscure. It has been reported that RA can modulate the expression of PA in several cell types; this activity being increased [15, 16] or reduced [17] depending on the cellular type (see also [12] and [18]).

Therefore, as a possible mechanism of its teratogenicity, we have investigated the effect of RA on the PA activity of post-implantation mouse embryos. In preliminary studies we have characterized the time-course of PA activity during early post-implantation stages of mouse embryos. Then, the effect of RA has been studied in embryos exposed to teratogenic doses of RA *in vitro* and *in vivo*.

MATERIALS AND METHODS

Reagents. All-trans RA, amiloride and urokinase (u-PA) were obtained from the Sigma Chemical Co. (St Louis, MO). RA was stored in the dark in DMSO (25 mg/mL). The plasmin chromogenic substrate S2251 and plasminogen were purchased from Kabi Vitrum (Brussels, Belgium). Tissue-type plasminogen activator (t-PA) was a gift from Prof Collen (KUL, Belgium). All other reagents were from Merck (Darmstadt, F.R.G.).

Animals. NMRI mice were used. The day 0 of gestation was defined as beginning at the midpoint of the dark cycle during which copulation took place.

The females were checked for vaginal plug the following morning. For assessing the change in PA activity during embryogenesis, the embryos were removed at different ages of gestation. The number of somites was counted and each embryo was transferred in a tube containing 500 μL Tris–HCl 0.1 M, pH 7.4 and homogenized by sonication (Virsonic 300, 10% intensity during 30 sec).

In vitro *effect of RA.* For *in vitro* experiments, the mouse embryos were explanted on day 8.5 of gestation. In this case, removal of uterine decidua, parietal endoderm and Reichert's membrane was performed in the culture medium; the ectoplacental cone was preserved. Pooled embryos from 2 to 3 mice displaying 5 to 9 somites were cultured in heat-inactivated human (80%) and rat (20%) serum according to the method previously described [19, 20]. RA was added in a final volume of 25 μL.

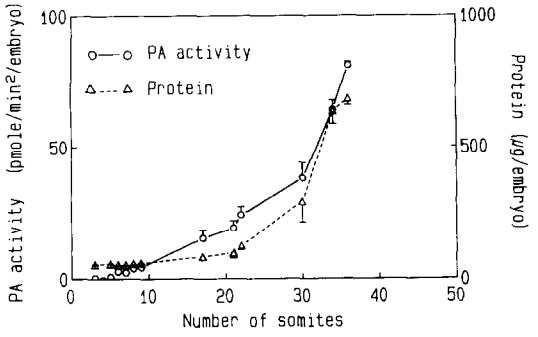

Fig. 1. *In vivo* evolution of PA activity in mouse embryos. Each point represents the mean ± SD of 3–4 embryos.

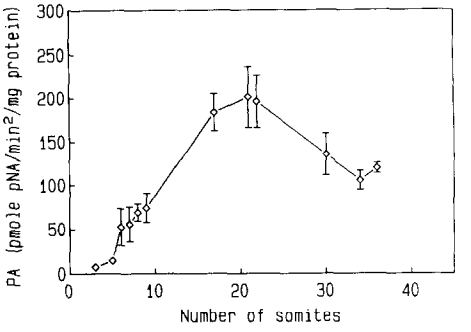

Fig. 2. *In vivo* evolution of specific PA activity in mouse embryos. Each point represents the mean ± SD of 3–4 embryos.

The controls received equivalent volume of vehicle. Final concentration of DMSO in the medium was always below 0.1% (v/v). After 0.25, 1, 6, 12 or 24 hr the cultures were terminated. The embryos were then examined for confirming the presence of yolk sac blood circulation and membranes were removed. The number of somites was counted and the embryo homogenates were prepared as described above.

In vivo *effect of RA*. On day 8.5 of gestation, the animals of one group (N = 3) received a single intraperitoneal injection of retinoic acid at a teratogenic dose (20 mg/kg; 200 µL) whereas those of the other group (N = 3) (control) were injected with a similar volume of the solvent (DMSO); 24 hr after the injection, the mice were killed and the embryos removed. After dissection of the membranes, each embryo was homogenized as above. For each mouse, 5 embryos were randomly selected for determining the number of somites, their PA activity and their protein content.

Measurement of proteins and PA activity. The protein content of the embryos was measured by a modification of the method of Lowry *et al.* [21]. PA activity was determined using 0.7 mM S-2251 and 0.165 CU/mL plasminogen in Tris–HCl 0.1 M pH 7.4. The acceleration of *p*-nitroaniline (pNA) production was monitored at 405 nm as described by Drapier *et al.* [22]. In some experiments amiloride was added at a final concentration of 10^{-3} M; u-PA and t-PA were used as reference substances.

Table 1. Inhibition of embryo PA activity by amiloride

	% Inhibition
References	
uPA	93.45 ± 0.50*
tPA	1.60 ± 3.00
Embryos	
8 somites	72.70 ± 16.70
21 somites	91.33 ± 0.70
35 somites	85.37 ± 3.10

* Mean ± SD of four determinations.
Amiloride (final concentration 10^{-3} M) was added before the substrate.

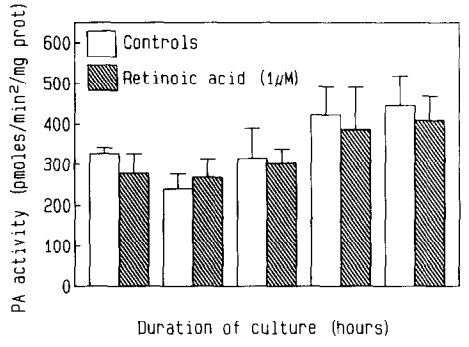

Fig. 3. *In vitro* effect of retinoic acid on mouse embryo PA activity. Each bar represents the mean ± SD of 4 embryos.

RESULTS

Development of PA activity in post-implantation embryos in vivo

PA activity was measured in post-implantation embryos *in vivo* displaying 3 to 36 somites. Total PA activity, total proteins and PA activity normalized for protein content at various developmental stages are presented in Figs 1 and 2. Total PA activity and as expected total protein content increase with the number of somites; the specific PA activity reaches a maximum at 21 somites and then declines.

In order to determine the type of PA activity expressed in mouse embryos, measurements were performed in the presence of the urokinase-specific inhibitor amiloride [23]. The results are presented in Table 1. Whatever the stage of the embryo examined, the main activity is of the urokinase-type.

In vitro *effect of RA on embryonic PA activity*

In preliminary experiments, a micromolar concentration of RA was found to cause marked malformations without affecting the viability of the embryos as evaluated by the presence of a yolk sac circulation.

PA activity was measured after exposing embryos to RA (10^{-6} M) for 0.25, 1, 6, 12 and 24 hr. Results are shown in Fig. 3. RA did not significantly influence

specific PA activity. This lack of effect has been observed at each incubation time up to 24 hr (Student's *t*-test, $P > 0.05$). After 24 hr of exposure to RA the embryos showed malformations and incomplete turning was observed in 75% of exposed embryos.

In vivo *effect of RA on PA activity*

PA activity was determined in embryos explanted 24 hr after treatment of the dams with RA; all these embryos were malformed. The specific PA activity was not significantly different between the treated and the control groups (respectively 87.3 ± 29.9 vs 92.0 ± 24.1 pmol pNA/min^2 mg protein, $N = 15$).

DISCUSSION

PA activity in post-implantation embryos

The extensive degradation of proteins that accompanies tissue modeling during embryogenesis has led to the suggestion that proteases may play a crucial role in development [10]. It has been shown that PA is secreted by different tissue layers [24] during mouse embryogenesis between day 7 and day 10 of development [11]. Parietal endoderm, portions of the visceral endoderm, mesoderm and ectoderm underlying the embryonic region of the egg cylinder and the extra-embryonic portion of the visceral endoderm produce PA [11]. Strickland and co-workers have also demonstrated that parietal endoderm produces PA. This enzyme may participate in the migration of the cells and/or in the metabolism of Reichert's membrane which accompanies embryo growth [8]. As shown in the present study and also as previously reported by Bode *et al.* [11], not only the external layers but also the intrinsic tissues of the embryo contain PA. Specific PA activity increases in the embryo from stage 3 (8.5 days) to 21 somites (9.5 days). This period closely coincides with the major events in organogenesis. Since PA production has been shown to be associated with migration [1–7] and accelerated growth [25, 26] in different cell types, it is not surprising to observe an increased PA activity during this period of development.

Marotti *et al.* [10] have shown that visceral endoderm and extra-embryonic mesoderm produce a different plasminogen activator type (u-PA) than parietal endoderm (t-PA). The results obtained with the inhibitor amiloride lead us to conclude that the main PA activity in the embryo is of uPA-type. It is interesting to note that cells endowed with migratory and invasive properties such as monocytes–macrophages, polymorphonuclear leukocytes and most malignant cells synthetize and secrete uPA [1–7]. The embryo, in which extensive cell migration is the rule, also mainly expresses a uPA-type activity.

Effect of RA on embryonic PA activity

It is known that RA can modulate plasminogen activator activity in a number of cultured cell types [5]. For example, it has been reported that RA stimulates PA synthesis in chick embryo fibroblast cultures [15] and chick embryo muscle cells [16]. We have recently shown that RA can inhibit *in vitro* the induction of plasminogen activator in macrophages stimulated by phorbol 12-myristate 13-acetate [17]. The teratogenic action of RA is well established [13, 14, 27–29], but, its mechanism of action is still unsettled. Recent findings suggest that RA may exert its action by altering gene expression through binding to specific intracellular proteins and direct interaction with nuclear DNA. The role of a receptor cascade system involving protein kinases in the transduction of extracellular signals into intracellular events has also been emphasized (see Ref. [30]). A possible effect on cell migration has been suggested [13, 27]. The latter might be determined by modulation of PA synthesis or modification in glycosylation reactions and alteration of cell surface glycoconjugates [12]. In order to determine if the PA modulating capabilities of RA are involved in its teratogenic activity, we have measured the PA activity of post-implantation mouse embryos exposed *in vitro* or *in vivo* to teratogenic doses of this agent. The results show that there is no significant differences in PA activity between the control and the exposed embryos. Since global PA activity was measured, the possibility that RA has nevertheless interfered with PA activity at some critical sites leading to malformations cannot be formally excluded. This hypothesis however seems unlikely since RA induces polymorphic malformations involving different cellular types. Moreover RA is a directly active teratogen capable to diffuse throughout the embryos. Therefore, if PA had been a target molecule for RA, this would have been detected in the present study. In conclusion our results suggest that interference with PA activity in the embryo is not the mechanism responsible for the teratogenic activity of retinoic acid.

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