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The Antibody-neutralisation of PDGF, CSF-1, TGFb2,3, EGF and EGF-receptor *in utero* in Pre-implantation Mice

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ABSTRACT

The aim of this study was to determine whether specific growth factors, those shown to be involved using PCR and immunohistochemistry, are necessary for the in-vivo mechanisms of normal implantation in mice.The abdomen of pregnant female mice were opened surgically on day 4 to expose the uterine horns, which were microinjected with specific neutralising antibodies against PDGF, CSF-1, TGFb2,3, EGF and EGF-receptor. At autopsy on day 12, the numbers, positions and sizes of all implantation and resorption sites were recorded. Sham-operation controls were utilised to evaluate the implantation model.Normal female mice exhibited a mean of 6.24 implantation sites per uterine horn. Sham-operated mice exhibited a 30.8% reduction in implantation compared with normals, and saline-injected mice exhibited a 45.7% reduction. Antibody-injected horns were compared with horns injected with saline and horns injected with heat deactivated antibody. All neutralising antibodies tested resulted in significant reductions in the implantation rate and the size of the implantation site. These experiments confirm, *in vivo*, participation of the specific growth factors tested in the mechanisms of murine implantation, as alluded to previously by evidence from PCR, *in vitro* stimulatory and immunohistochemical work.

INTRODUCTION

The gene expression of growth factors and their receptors in the preimplantation embryo and uterus of experimental animals (18,19,21) have been determined by a number of investigators using the polymerase chain reaction (PCR) with specific identification probes, despite the minute amounts of tissue involved. Nevertheless, the presence of the growth factor or receptor RNA only confirms the possibility of production, release and functioning of the gene product. This problem has been overcome to a limited extent in reproductive tissues by immunohistochemical identifications (5,13) or by the stimulation of preimplantation embryos with the factors *in vitro* (4,14,15,19). However, very little work has been done *in vivo* to demonstrate whether these growth-stimulating, gene products are directly involved in the mechanisms of blastocyst activation, hatching and implantation.

The work documented here presents *in vivo* evidence that the activities of specific growth factors previously detected by the PCR-technique are significantly involved in the

mechanisms of successful and normal implantation *in vivo* in the mouse. The *in vivo* mouse model utilized involves micro-injection of each antibody separately into one uterine horn and the vehicle into the contralateral horn as an intra-animal control. Results show that each relevant antibody tested, i.e., those with published evidence of gene expression, exhibited a specific inhibition or reduction of the implantation rate.

MATERIALS AND METHODS

Random-bred NMRI mice were maintained under controlled lighting to provide a 10 h night centred on midnight, and received a standard diet and fresh water *ad libitum*. Female mice weighing 25 to 35 g were individually mated with proven fertile males and successful mating was confirmed by the presence of a vaginal plug between 8 and 9 *am.*, this being considered day 1 of pregnancy.

On day 4 of pregnancy after 2 *pm.*, the mice were anesthetized with a 3 to 1 solution of 50 mg/mL Ketalar^{TM} (Parke-Davis SA, Barcelona) and 20 mg/mL Rompun vet.^{TM} (Bayer AG, Leverkusen), respectively. The abdominal hair was removed, the abdomen was opened suprapubically with a mid-line incision, and the overlying fat and bladder were exteriorized to provide a clear view of the bicornuate uterus and cervix. A ligature was placed around the utero-cervical junction without incorporating the mesometrial vessels in order to obtain a non-patent lumen without causing necrosis. Thereafter, a Hamilton microsyringe was used to inject 5 uL of fluid into the upper lumen of each uterine horn. One horn received vehicle only and functioned as the control side, while the contralateral horn received vehicle plus antibody and functioned as the experimental side. The choice of horn for control and experimental was randomized.

Five different antibodies were tested for their ability to inhibit implantation, namely antibodies against **PDGF** (1mg/mL. British Biotech Ltd., UK.), **CSF-1** (monoclonal rat anti-CSF-1. Lot no. 89900-105, 100ug/mL. Oncogene Science Inc., N.Y.), **TGFb** (monoclonal murine anti-TGFb2,3. Lot no. 01450, 1mg/mL. Genzyme Corp., Cambridge, MA, USA.), **EGF** (polyclonal rabbit anti-murine EGF. Lot no. B9230, 1mg/mL. Genzyme Corp., Cambridge, USA) and **EGF-receptor** (monoclonal anti-EGF receptor. Lot no. 13, 100ug/mL. Amersham Int., U.K.). Each antibody was injected (5uL) in its original concentration and vehicle, and the denatured antibody controls had been incubated at 70^{o}C for 30 min.

Subsequent to the uterine horn instillations of antibody and vehicle or vehicle alone, the adipose tissue and bladder were returned to the abdomen which was closed using continuous 6-0 silk sutures both for the abdominal muscle layer and the skin. With this technique, the specific antibodies came in contact with the blastocyst and endometrium immediately prior to the time of blastocyst activation and hatching and the initiation of the decidual response, followed by implantation during the morning of day 5. These female mice, postoperatively, were returned to their respective males where they were maintained until sacrificed by cervical dislocation on day 12. At autopsy, the numbers, positions and sizes of all implantation sites were recorded, as were also the numbers of obviously resorbing implantations (*Table 1*).

An additional, sham-operation group of day 4 - pregnant, control mice were anesthetized and treated surgically in an identical manner to those described above except that

no fluids were injected when the micro-syringe needle was placed in the upper uterine lumen. The implantation rate in this sham-operation group (see *Table 1, group 11*) can be compared with the implantation rates in both the antibody-treated experimental horns and the vehicle-treated control horns.

Statistical differences between the groups in *Table 1* were calculated using Student's t-test corrected for small numbers of observations and, where relevant, for paired and intra-animal observations. A probability of 5% or less was considered indicative of a significant difference.

RESULTS

The animals used in these experiments were outbred NMRI mice with an average of 6.24 implantation sites per uterine horn normally, these being observed to vary from 7 to 10 mm greatest diameter on day 12 of pregnancy (*Table 1, group 1*). These are the basal values in the normal animal. In order to utilize this research animal as an experimental system, a non-necrotizing ligature was tied around the uterine cervix where the two horns merge to avoid leakage of fluid, and the uterine wall and endometrium must be penetrated with a needle in order to inject active antibody into the lumen. From *Table 1*, it can be seen in *group 2* that the ligature and the needle penetration together cause a direct 30.8% reduction in the average number of implantation sites per uterine horn, when compared to the normal pregnancy controls in *group 1* . Moreover, the injection of 5 uL of saline into the uterine lumen, as seen in *group 3*, disturbs normal implantation even more, reducing the number of sites by 45.7% compared to the *group 1* normals.

These drastic effects resulting from surgical preparation of the animal demonstrate the baseline of the experimental system and, as a consequence, additional intra-animal controls were run in the contralateral horns of all animals treated with neutralising antibodies, these being given directly or after denaturation. Therefore, any reductions in the implantation rate in experimental horns treated with active neutralising antibody should not only be compared with the baseline controls of group 3 but also with the intra-animal contralateral controls treated with antibody vehicle only, and with the experimental or control horns treated with denatured antibody or vehicle, respectively.

Utilising this strategy, PDGF antibodies resulted in a significant 50% reduction in the number of implantation sites, while the denatured PDGF antibodies had no significant effect (3.8%) (*groups 4 and 5, resp.*). Furthermore, the PDGF antibodies also reduced the post-implantational growth of the sites, as seen by their sizes in the 13 experimental horns of *group 4*, compared with the control horns in the same *group* and in *group 5.*

In addition, Table 1 shows that the neutralising antibodies for CSF-1, TGFb2,3, and EGF also significantly reduced the implantation rate by 64.7%, 76.5%, and 17.3%, respectively, compared with their contralateral controls and their denatured antibody counterparts. It should also be noted that the size range of the implantation sites in *groups 4* to *11* are approximately the same as that of the *group 3* baseline controls, with the exception of *group 8* which is more within the normal range.

Preliminary data from 6 animals injected with an EGF-receptor antibody which was dissolved in phosphate buffered saline plus 1% bovine serum albumin (BSA) exhibit an

Table 1. Implantation rate after *in-utero* injection of growth factor antibody, including the respective vehicle and denatured controls, in NMRI mice.

Group No.	No. mice	Uterine horn	Treatments	No. of impl'ns	Size range mm	Impl'ns per uterine horn	% reduced impl'n rate
1	19	38 control horns	**Normal bilateral pregnancies**	237	7 - 10	6.24	0.0 %
2	11	22 control horns	**Sham operation**, needle penetration, no injection	95	7 - 10	4.32	30.8 %
3	14	28 control horns	**Sham operation**, needle penetration, 5 ul saline injection	95	3 - 10	3.39	45.7 %
4	13	Control	5 uL saline	44	4 - 9	3.38	
	13	Exptl	Anti-**PDGF** in 5 uL saline	22	2 - 7	1.69 *	50.0%**
5	8	Control	5 uL saline	27	4 - 11	3.38	
	8	Exptl	Denat. anti-**PDGF** in 5 uL saline	26	4 - 11	3.25	3.8%**
6	15	Control	5 uL PBS	51	3 - 9	3.40	
	15	Exptl	Anti-**CSF-1** in 5 uL PBS	18	3 - 9	1.20 *	64.7%**
7	8	Control	5 uL PBS	23	3 - 9	2.87	
	8	Exptl	Denat anti-**CSF-1** in 5uL PBS	22	3 - 9	2.75	4.2%**
8	12	Control	5 uL PBS	51	7 - 9	4.25	
	12	Exptl	Anti-**TGFb** in 5 uL PBS	12	2 - 9	1.00 *	76.5%**
9	5	Control	5 uL PBS	12	4 - 9	3.60	
	5	Exptl	Denat anti-**TGFb** in 5 uL PBS	11	3 - 9	3.30	9.2%**
10	26	Control	5 uL PBS	87	3 - 9	3.35	
	26	Exptl	Anti-**EGF** in 5 uL PBS	72	2 - 9	2.77 *	17.3%**
11	9	Control	5 uL PBS	34	4 - 9	3.78	
	9	Exptl	Denat anti-**EGF** in 5 uL	33	2 - 9	3.69	2.4%**

* Significant difference compared with **Sham-operated** and **Denatured antibody** controls ($P<0.05$).
** Percent reduction in implantation rate compared with **Group 3, Sham-operated** controls.

Mean implantation rate in the contralateral uterine horn controls is 3.45 (n = 102).
Mean implantation rate in the denatured antibody controls is 3.25 (n = 30).

implantation rate of 2.83 per horn for the contralateral controls and 0.33 per horn in the experimental group. This represents an 88.3% reduction in the implantation rate. However, the contralateral control horns exhibited a 16.5% reduction in their implantation rate compared with baseline controls, a variation which may be due to the 1% BSA in the antibody vehicle, since BSA is an active biological compoundand could affect murine implantation.

DISCUSSION

These experiments represent an attempt to confirm, *in vivo*, the participation of specific growth factors in the mechanisms of murine implantation, growth factors which have previously been implicated by PCR, *in vitro* stimulatory, and immunohistochemical evidence. The *in vivo* data above support the previous findings with regard not only to the production but also the involvement of PDGF, CSF-1, TGFb2 or 3 and EGF in blastocyst implantation and the establishment of pregnancy.

The percentages by which the implantation rate is reduced in *groups 2* and *3* (30.8% and 45.7%, resp.) define the effects of preparing the animal model, and the implantation rate of 3.39 per uterine horn for *group 3* must be considered as the baseline for comparisons. As such, the *group 3* implantation rate can be compared with the contralateral horns of *groups 3* to *11*, where the mean implantation rate of 3.45 (n = 96) is not significantly different, all other factors considered to be constant. When the implantation rate in the contralateral horn has varied significantly from that in the baseline controls, this has usually meant that the active compounds injected into the experimental uterine horn are leaking into the vascular circulation and, thereby, affecting the contralateral horn controls, either negatively or positively (unpublished data). The data from these experiments suggest that the antibody solutions are not affecting the contralateral control horns.

PCR-detection has previously indicated gene expression for PDGF, TGFa, TGFb and IGF-II, but not for FGF, EGF, NGF, G-CSF, IGF-I or insulin, in murine preimplantation embryos (17, 21). In addition, antigens for PDGF, TGFa and TGFb have been detected in blastocysts by immunohistochemistry (17). Furthermore, 2-cell mouse pre-embryos cultured in microdrops exhibited markedly improved development when stimulated *in vitro* with either EGF, TGFa, TGFb1 or EGF plus TGFb1,while IGF-I had no additive effect on EGF-induced stimulation (15). This combined EGF-TGFb growth stimulating effect on mouse pre-embryos (15) has been observed previously on normal rat kidney fibroblasts growing in soft agar (2, 3). Additional corroborating evidence for the requirement of specific growth factors for pre-embryo development and implantation comes from work with bovine embryos which exhibit gene transcripts for PDGF-A and TGF (24).

Maternally encoded mRNA for PDGF-A chain is expressed in Xenopus embryos up to the stage of mid-blastula transition (12). PDGF-A chain transcripts (PCR) and antigens (immunohistochemistry) are present in murine preimplantation stages (17, 21), while the transcripts have also been detected in bovine pre-embryos (25). In addition, PDGF-B chain antigens were demonstrated by radioimmunoassay (RIA) in culture media from human blastocysts (23). Consequently, it seems safe to say that the preimplantation conceptus produces and releases at least one of the PDGF isoforms. However, there is no evidence yet to

suggest that an autocrine effect may exist; Colver and coworkers in 1991 (1) observed no growth or development of preimplantation mouse embryos in a defined medium after stimulation with human PDGF.

Svalander and colleagues in 1991 (23) detected by RIA significant levels of PDGF in samples of human uterine secretions from both the follicular and luteal phases. Also in 1991, Surrey and Halme (22) demonstrated that purified human PDGF stimulated the incorporation of tritiated thymidine in cultures of endometrial stromal cells from the proliferative phase. So it seems that PDGF is secreted by cells of the endometrium, probably macrophages since both PDGF and macrophages are involved in the inflammatory response associated, for example, with endometriosis (20, 11).

PCR detection has been unable to disclose the expression of EGF in all preimplantation stages of murine (21) or bovine (25) embryo development. Therefore it seems generally unlikely that EGF is produced and released by preimplantation stages. However, the present work does demonstrate an involvement of EGF in the latter stages of development and implantation. Moreover, it appears that murine pre-embryos can be stimulated in vitro by EGF, thereby improving their development to the blastocyst stage, increasing the number of trophoblast cells per blastocyst, and enhancing the rate of zona hatching (15-16). The neutralising antibodies against molecules of EGF and EGF-receptor in the present results were quite effective in inhibiting the *in vivo* implantations, demonstrating the involvement of this growth factor in the implantation process. Added to this, the autoradiographic localisation of cell-surface EGF binding on 8-cell, morula and blastocyst stages strongly suggests the presence of receptors (15), receptors which are able to bind both EGF and TGFa.

Since TGFa transcripts and antigens have been demonstrated by PCR detection in blastocysts and antigens by immunohistochemistry from as early as oocytes through to blastocysts (17, 21), and since the endometrium produces both EGF and TGFa, then the systematic growth factor effects described by Paria and Dey in 1990 (15) are most feasible.

The uterine luminal epithelium and the conceptus have cell membrane EGF/TGFa receptors. The conceptus produces TGFa from an early stage and, thereby, can stimulate its own development autocrinologically, while also communicating its presence paracrinologically to the epithelial cells in the uterine lumen, a possible conceptus-maternal message. In the meantime, the EGF/TGFa receptors in both places are stimulated to develop by ovarian steroids (24), a development involving the steroids in a very specific ratio and most likely demanding rapid activation of tyrosine aminotransferase at the trophoblast cell membrane (6). Growth factor ligand binding to the conceptus then results in protein tyrosine kinase activity and rapid autophosphorylation of the receptor. An additional important relationship here is that EGF stimulates the release of prostaglandin E2 (PGE2) from cultured human endometrial cells (9), while PGE2 has been associated with murine implantational events (7) and seems to be released by human preimplantation embryos (8).

An additional factor, or system, which may be involved in the inplantation inhibiting effects seen in these results could be the complement system (10). This system could possibly be activated by the immuno-complexes arising from the intra-uterine antibody injections. However, this was not investigated in this report.

In conclusion, our data confirm that PDGF, CSF-1, TGFb and EGF are actively involved in the implantation process *in vivo*. Whether the antibody neutralisations

demonstrated were due to an effect on the embryo, on the endometrium, or on both, remains to be established.

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