Vol. 198, No. 1, 1994
January 14, 1994
BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 150-157

TRANSIENT THIOL-EXPRESSION AND THIOL DISULPHIDE CLUSTER-RECEPTOR COMMUNICATIONS : A MOLECULAR BASIS FOR BLASTOCYST-UTERINE INTERACTIONS DURING IMPLANTATION

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Received November 22, 1993

The attachment of trophoblast to uterine epithelium during implantation is an intricate and complex instance of cell-cell communication, the molecular aspects of which remain rather undefined. In this study, we screened the surface domains of the pre-, peri- and post implantation mouse embryos with a thiol-specific probe. Transient thiol-expression in peri-implantation embryos suggested their involvement in implantation either through disulphide bridge establishment or through a communication between a thiol-disulphide cluster of the blastocyst and a corresponding receptor of the endometrium.

Cell adhesion, a universally observable phenomenon in multicellular organization, involves the establishment of loose attachment between two cells or cell and a substratum. The organization of animal cells in differentiated tissues and organs has long been postulated to depend on cell-surface interactions both with molecules on the surface of other cells and with extracellular matrix. Rigorous research over the years to identify the interacting surface molecules in various model systems has led to the characterization of a host of membrane proteins designated now as `adhesion molecules' acting as recognizing and anchoring entities. Members of this superfamily protein include the L-CAM, N-CAM and the cell-CAM 120/80 [1].

0006-291X/94 $5.00

Implantation of trophoblast in the uterus presents a unique instance of cell-cell communication and subsequent adhesion defining the fate of a conceptus. In the mouse, during implantation, the trophoblast attachment involves the whole of the outer surface of the cells, in contrast to some other species where it first attaches to the endometrium by trophoblastic knobs or projections [2]. The trophoblast-uterine epithelial contact sites are important for cellular communication and function perhaps to initiate embryonic development and/or to induce cell transformation. The sialomucins on the surface coat are probably changed, since there is a change in the surface charge of the blastocyst, the active blastocyst is less negative than the inactive ones [3]. This charge is probably a result of intracellular changes in the configuration of the surface of the uterine epithelium, the regular microvilli disappear and surface attains a more smooth but undulate appearance.

In trying to understand the adherence phenomenon during implantation, all efforts have been directed towards probing the accessible saccharide moieties on the blastocyst during the course of its development. The external and internal surface of zona pellucida reveal variable lectin-binding property [4]. Differentiation of the blastocyst trophoblast during the peri and early post implantation period was associated with a differential loss of lectin receptors in all cell lineages of the mouse conceptus [5].

A new idea emerges defining the `reactive groups' of the biomembrane surfaces as `communication terminals' in various cell interaction models. With pre- and peri-implantation trophoblasts as models, we examine the sequential expression of surface-thiols, by mouse embryos and its possible involvement in preliminary communication reactions.

MATERIALS AND METHODS

ANIMALS: Mature inbred female mice (Swiss strain 3-4 months old) housed in temperature controlled (27 $\pm 1^{o}$C) rooms at light : dark regimen of 14:10 hr. were used for the studies.

REAGENTS: (5-IAF) 5-iodoacetamidofluorescein was obtained from Molecular probes, MI, USA.(NEM) N-Ethylmaleimide was purchased from Sigma chemical company USA. Hanks balanced salt solution (HBSS) was made of reagent grade chemicals, pH was adjusted to 7.2 by using 50 mM, N-2-hydroxy ethyl piperazine-N'-ethane sulphonic acid (HEPES) [6] obtained from Hi-Media, Bombay, India.

BLASTOCYST COLLECTION: Blastocysts were collected from naturally mated female mice. Female mice which showed a regular 4 or 5 day estrous cycle were used. Vaginal smears were examined daily by the method of Stockard and Papanicolau [7]. Only those female mice which showed a proestrous smear were mated with a male mice of proven fertility, the same evening. The presence of vaginal plug on the following morning confirmed mating. The day of mating was designated as day 1, while the day of proestrous was designated as day 0.

Blastocysts of day 4 (5.00 p.m.), day 5 (5.00 a.m.) and day 5 (10.00 a.m.) were collected by flushing the uterus with HBSS (pH 7.2) into a watch glass. The blastocysts were subjected to the histochemical assay for thiol localization. For each day of pregnancy, 5 mice were sacrificed, both uterine horns were flushed out and blastocysts were subjected to 5-IAF binding as outlined below.

5-IAF BINDING : Initially, 5-IAF binding technique was standardized using a concentration range of 5-IAF from 10 µg/ml-200µg/ml with various reaction time viz. 5min., 7min., & 15min. The optimum concentration and optimum time was found to be 20µg/ml and 7 min, respectively.

Blastocysts, collected in watch glasses were incubated with 5-IAF (20µg/ml in double distilled water and ethanol in the ratio of 1:1) for 7.00 min in dark in the reagent solution having HBSS buffer and 5-IAF in the ratio of 75:25. The pH of the reagent solution was adjusted to 7.2. After incubation the blastocysts were transferred to another watch glass containing fresh HBSS. The changes of the buffer (2ml each) were made to remove unreacted fluorophore.

Controls for each experimental set were made by pre-treating the blastocysts for 10 min with N-Ethylmaleimide (200µg/ml made in HBSS, pH adjusted to 7.2). N-Ethylmaleimide is known to be a perfect blocker of free sulphydryl by forming covalent bonds with sulphydryl groups at pH 6.5-7.5 [8]. The blastocysts were washed twice to remove unreacted NEM by transferring them into watch glasses containing fresh HBSS buffer and were subsequently processed for 5-IAF binding as described earlier.

The blastocysts were observed under NIKON OPTIPHOT TMD inverted microscope equipped with phase contrast acessories. Photographs were taken using ORWO NP 27 (ASA-125) black and white films. Exposure time was computed by NIKON UFX II Camera monitor unit. Films was processed using fine grain developer and Amfix fixer (May and Baker, India).

RESULTS

After a series of experiments to define the exact time of implantation in our model system, it was found that in Mus musculus implantation occurs around 5 a.m. to 6 a.m. on day 5. Thereafter, we restricted our study to three model groups: (1) Pre-implantation group, where the blastocysts were harvested on day 4, 5 p.m; (2) Peri-implantation group, where they were harvested on day 5, 5.00 a.m.; and (3) Post-implantation group, where the blastocysts were harvested on day 5, 10.00 a.m. The last group signified the blastocysts which failed to establish implantation during the normal time span.

On day 4 (5.00 p.m.), the blastocysts were generally unhatched. The zona pellucida stained faintly with 5-IAF at this stage. 5-IAF crossed the zona pellucida layer and stained the blastocyst surfaces. The blastocyst surface staining was also weak, but patchy at this stage (Fig. 1, a & b). The 5-IAF binding was totally inhibited by a pre-treatment of the blastocyst with N-Ethylmaleimide (Fig. 1, c & d).

Day 5 (5 a.m.) blastocysts were generally hatched. The thiol-localization was intense on the equatorial domain of the blastocyst surfaces. (Fig. 1, e & f). Again, this pattern was abolished by N-ethylmaleimide, showing the thiol-specificity of the fluorescence probe used. (Fig. 1, g & h). The post-implantation blastocyst showed very weak 5-IAF binding (Fig. 1, i & j). The corresponding NEM-treated blastocyst is also presented (Fig. 1, k & l).

DISCUSSION

Implantation is a process whereby the blastocyst becomes embedded in the endometrium. This involves three stages:

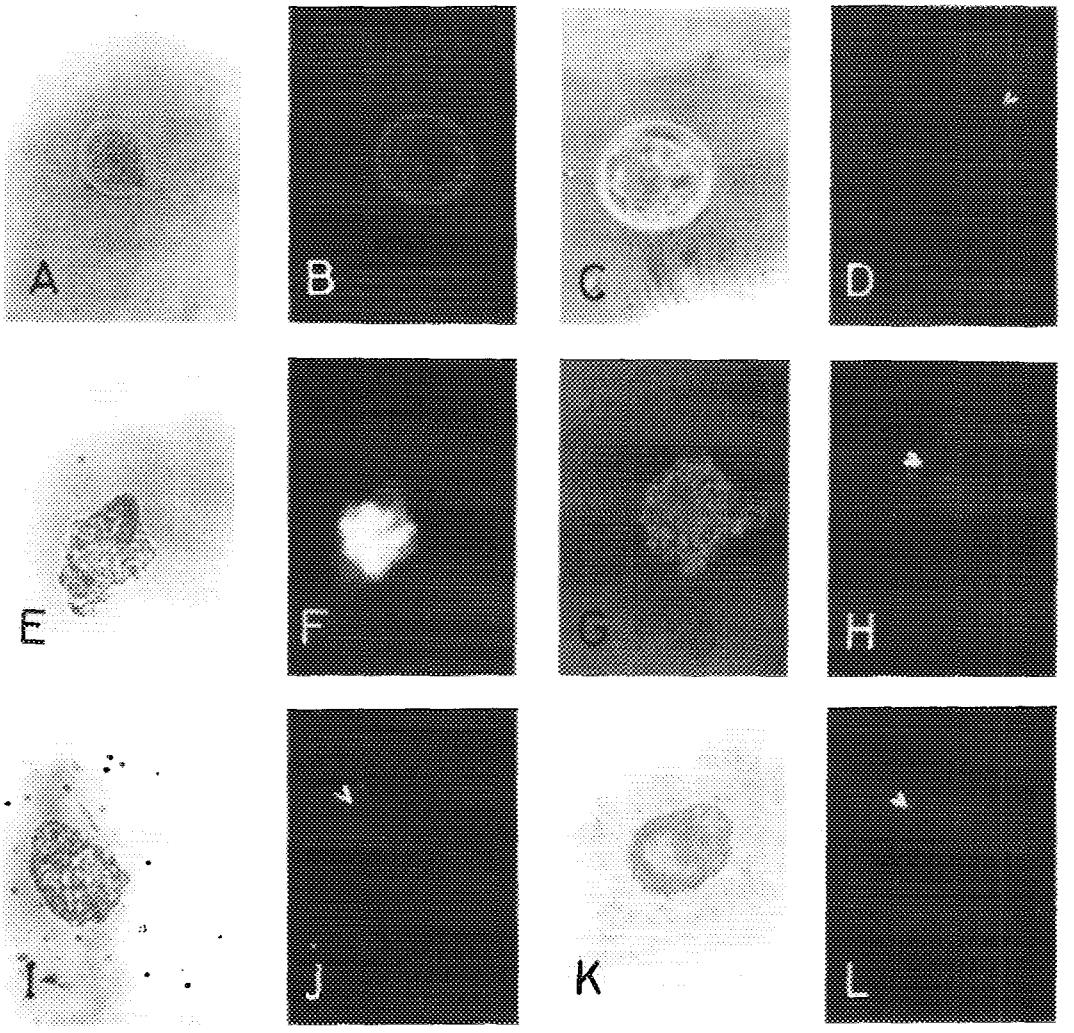

Figure 1. Photomicrographs of mouse blastocysts at various stages of development. B, F & J show the thiol-distribution on the surfaces of pre-, peri-, and post-implantation embryos, demonstrated using 5-iodoacetamidofluorescein (20µg/ml). A, E & I are their respective phase-contrast images.

D, H & L show the fluorescent photomicrographs of pre-, peri-, and post-implantation embryos labeled with 5-iodoacetamidofluorescein in the presence of a thiol-blocker N-ethyl maleimide (200µg/ml). The absence of fluorescence indicates the thiol-specificity of 5-IAF. C, G & K are the corresponding phase-contrast images. (x 120.)

Arrows in D, H, J & L indicate the blastocyst's location.

blastocyst activation, trophoblast attachment and trophoblast invasion [9]. The early trophoblast uterine epithelial contact sites are probably important for cellular communication and

function perhaps to initiate embryonic development and/or to induce decidual cell transformation. The initial apposition of the trophoblast and the uterine epithelium is brought about by the closure of the uterine lumen and to a lesser extent by the expansion of the blastocyst [9].

The blastocyst's trophectoderm is a polarized epithelium that can transport ions and secrete proteins [10]. Mouse blastocysts secrete proteins in the apical direction [11,12] and the proteins may be involved in blastocyst development [13] and/or implantation [14]. The presence of surface sialic acid residues in uterine epithelium and its possible involvement in implantation have been suggested [15]. Attempts to identify Cell-CAM superfamily proteins in uterine epithelium at the time of implantation have yielded negative results [16].

The uterine luminal proteins from Day 5 pregnant females have been shown to have a higher affinity for blastocysts than do uterine proteins from proestrous rats [17]. Competitive assays illustrated the specificity of this phenomenon. Even though the molecular aspects of this binding are not defined, the binding of luminal proteins to the embryonic surface might enhance specific adhesion at the onset of implantation [18].

While all theories laid down to explain blastocyst attachment to the uterine epithelium remain unaccepted, we founded an entirely new concept that cell-cell interaction(s) could be mediated by reactive thiols (-SH) on the surfaces. This hypothesis was validated using the mouse pre-, peri- and post-implantation models. The `thiol expression' over the blastocysts exhibited development related alterations.

Accumulation of thiols over the equatorial domains of the peri-implantation embryo, probably through their lateral mobility, suggests their possible involvement in implantation. The interacting counterpart could be a thiol or a receptor which could communicate with a re-arranged thio-disulphide cluster in a space-filling and non-competitive fashion.

ACKNOWLEDGMENT

This work was supported by the Department of Atomic Energy, Bombay, India, vide R/D project No. 4/9/92-G to ML.

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