Compartmentalized reactivity of M3/38 (anti-Mac-2) and M3/84 (anti-Mac-3) in the uterus of pregnant mice

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Changes in the expression of antigens on mouse uterine or embryonic tissues were examined by enzyme immunocytochemistry. Cryostat sections of uteri from days 1, 8 and 15 of pregnancy were probed with the monoclonal antibodies M3/38 and M3/84, originally defined by their reactivity with macrophage surface antigens (Mac-2 and Mac-3, respectively). In the uterus of pregnant mice, reaction of these antibodies was not limited to leucocytes. M3/38 did not react at detectable levels with cells in the uterus on day 1 but did react with decidual cells immediately surrounding the embryo on day 8. By day 15, the placenta, decidua basalis and metrial gland were intensely positive but the embryo was negative. M3/84 reacted with the luminal side of the endometrium on day 1, the entire decidual mass on day 8, and with all maternal and fetal tissues on day 15. M3/38 was detected in saline-soluble preparations of uterine tissue and had a molecular mass of approximately 32–35 kDa as determined by SDS–PAGE and western blot analysis. The pattern of expression of these molecules suggests a functional relationship to developmental changes that occur at the maternal–fetal interface.

Introduction

In mice the nidatory stimulus initiates changes in the uterine stroma resulting in formation of decidual tissue that completely surrounds the conceptus by day 5 of pregnancy (Finn and McLaren, 1967; Parr *et al.*, 1987). Highly vascularized tissues develop between the conceptus and mesometrial side of the uterus and the trophoblast differentiates to form the placenta where maternal cells and fluids come into direct contact with fetal tissue (Welch and Enders, 1987, 1991). In the mesometrial triangle, uterine stroma and other cells develop into the metrial gland. The functional significance of such compartmentalization is poorly understood and in most cases the regional molecular specialization that is associated with the compartments has not been clearly defined. In this study we report on differential expression of two immunologically distinct molecules, recognized by the monoclonal antibodies M3/38 and M3/84, that further define compartmentalized development of the mouse uterus during the early stages of pregnancy. The pattern of molecular specialization that was observed suggests a functional correlation with the events of implantation and placental development.

Materials and Methods

Animals and breeding

Outbred Swiss mice were used for all studies. Male and female mice were housed in separate rooms in an environment

with controlled temperature and humidity with 12 h light:12 h dark cycles. Females were placed with males overnight for mating. On the following morning mating was confirmed by detection of a vaginal plug (day 1 of pregnancy).

Tissue preparation

On days 1, 8 and 15 of pregnancy, mice were killed by cervical dislocation and uteri were removed and frozen in liquid nitrogen. Frozen tissues were embedded in Tissue Tek OCT compound (Miles Inc., Elkhart, IN) and sectioned at a thickness of 8 µm in a cryostat. Sections were air-dried, fixed in cold acetone, and stored at –20°C.

Cell lines and media

Hybridomas secreting rat monoclonal antibodies, M1/9.3.4.HL.2 (anti-common leukocyte antigen; immunoglobulin (Ig) G2a) (Springer *et al.*, 1978), M3/38.1.2.8.HL.2 (anti-Mac-2; IgG2a) (Ho and Springer, 1982), M3/84.6.34 (anti-Mac-3; IgG1) (Springer, 1981; Ho and Springer, 1983), 53-6.72 (anti-Lyt2; IgG2a) (Ledbetter and Herzenberg, 1979) and PC 61.5.3 (anti-interleukin 2 receptor; IgG1) (Ceredig *et al.*, 1985) were obtained from American Type Culture Collection (ATCC, Rockville, MD). The P388D1 macrophage cell line was also obtained from ATCC. All cells were grown in Dulbecco's modified Eagles medium (DMEM) containing 1500 mg glucose l⁻¹, 10 U penicillin ml⁻¹, 10 µg streptomycin ml⁻¹, and 5% fetal bovine serum.

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Fig. 1. Uterus of day 1 pregnant mouse stained with (a) M1/934, (b) media control, (c) M3/38, (d) 53-6.72 (immunoglobulin G2a isotype control), (e) M3/84 and (f) PC-61.5 (immunoglobulin G1 isotype control). Scale bar = 1 mm.

Immunocytochemistry

Culture supernatants from rat–mouse hybridomas were used as a source of monoclonal antibody. Exhausted media were centrifuged to remove cells and debris, filtered through 0.2 μm membranes, brought to 0.1% sodium azide, and stored at -20°C . Acetone fixed sections were hydrated in PBS for 30 min then placed in monoclonal supernatant for 2 h at room temperature. For day 8 longitudinal sections, acetone-fixed slides were placed directly into 2% paraformaldehyde for 5 min, then rinsed four times with PBS over 30 min, and placed into monoclonal supernatant for 2 h. Slides were rinsed with four changes of PBS for 10 min and placed into alkaline phosphatase-conjugated goat anti-rat IgG (no crossreaction to mouse IgG; diluted 1:200 in PBS, containing 1% BSA and 0.1% sodium azide; Cappel, Durham, NC) for 1 h at room temperature. Slides were again rinsed four times with PBS for 10 min before being placed in substrate (30 mg Fast Red TR, 6 mg naphthol AS-BI-phosphate (Sigma, St Louis, MO), 7.2 mg levamisole in 30 ml 0.1 mol Tris buffer l^{-1} , pH 8.2) for 30 min at room temperature. Sections were lightly counterstained with Mayer's haematoxylin then covered and preserved with Crystal Mount (Biomedica Inc., Foster City, CA).

Preparation of uterine and placental homogenates

Uteri were removed from pregnant animals on day 15 of pregnancy and opened along the anti-mesometrial side. Embryos were removed from the placentae with scissors and the placentae were gently peeled away from the uterus with forceps. Uteri and placentae were separately rinsed three times in ice-cold PBS, weighed, then homogenized at low speed in 0.5 ml cold PBS per 0.1 g tissue with a Brinkman homogenizer (Brinkman Instruments Co., Westbury, NY). The homogenate was centrifuged at 1200 g for 20 min to remove cells and debris. The supernatant was frozen at -20°C , thawed and centrifuged again. The clarified supernatant was sterile filtered through 0.2 μm filters and stored at -20°C .

SDS-PAGE western blot analysis

SDS-solubilized P388D1 cells and PBS-soluble extracts of day 15 uterus and placenta were separated on 10% Total (% Total: acrylamide + bis-acrylamide/100 ml), 2.7% Crosslinker (% Crosslinker: bis-acrylamide/total acrylamide $\times$ 100) SDS-PAGE under non-reducing conditions and electrophoretically

Fig. 2. Uterus of day 8 pregnant mouse, cross-sectioned through embryo and decidual mass, stained with (a) M1/934, (b) media control, (c) M3/38, (d) 53-6.72 (immunoglobulin G2a isotype control), (e) M3/84, and (f) PC-61.5 (immunoglobulin G1 isotype control). M: mesometrial connective tissue; D: decidual mass; E: embryo. Scale bar = 1 mm.

transferred to nitrocellulose (Laemmli, 1970; Towbin *et al.*, 1979). After transfer, blots were rinsed several times with PBS then rocked overnight at 4°C in PBS containing 2% BSA and 0.1% sodium azide. The next day, blots were placed into monoclonal supernatant and rocked at room temperature for 2 h. Blots were rinsed four times, and rocked for 30 min with changes of PBS every 10 min. Alkaline phosphatase conjugated goat anti-rat IgG (diluted 1/1000 in PBS, 2% BSA; Cappel, Durham, NC) was added and incubated at room temperature for 1 h. Blots were then rinsed as before and placed into substrate solution (6 mg naphthol AS-BI phosphate, 30 mg Fast Red TR, 7.2 mg levamisole, 30 ml 0.1 mol Tris l⁻¹, pH 8.2) until bands developed.

Results

Monoclonal antibodies M3/38 and M3/84 were tested for their ability to react with determinants on uterine or fetal tissues

during different stages of pregnancy. The anti-common leucocyte antibody, M1/934, was used to distinguish cells of leucocyte lineage from other tissues. Isotype matched monoclonals 53-6.72 (IgG2a) and PC 61.5 (IgG2b) were used as controls. Frozen sections from days 1, 8 and 15 of pregnancy were assessed for reactivity with each antibody.

On day 1 of pregnancy (Fig. 1) M1/934 reactive cells were distributed throughout much of the endometrial stroma and connective tissue (Fig. 1a). M3/38 reactivity was not detected in cells of the day 1 uterus (Fig. 1c) but was occasionally seen in low amounts in glandular fluids of some sections (not shown). M3/84 reacted intensely with the luminal side of the endometrium (Fig. 1e). By day 8 (Fig. 2), with the development of decidual tissue, M1/934-reactive leucocytes were heavily concentrated in the decidual mass between the implanted conceptus and the mesometrial side of the uterus (Fig. 2a) and scattered throughout the uterus, mesometrial connective tissue, and other areas of the decida. Although M3/38 did not react with most of the decidual tissue, uterus or mesometrial connective tissue, the area of the decidual tissue immediately adjacent to the conceptus

Fig. 3. Day 15 pregnant uterus stained with (a) M1/934, (b) media control, (c) M3/38, (d) 53-6.72 (immunoglobulin G2a isotype control), (e) M3/84, and (f) PC-61.5 (immunoglobulin G1 isotype control). MG: metrial gland; DB: decidua basalis; S: spongiotrophoblast region of the placenta; L: labyrinth region of the placenta; F: fetus. Scale bar = 2 mm.

was intensely positive (Fig. 2c). M3/84 reacted intensely with the entire decidual mass and to a lesser degree with the uterus and mesometrial connective tissue (Fig. 2e).

By day 15, M1/934 positive cells were concentrated primarily in the metrial gland (Fig. 3a) and just outside Reichert's membrane along the anti-mesometrial side of the uterus. These cells were also dispersed throughout the mesometrial connective tissue and myometrium (Fig. 4a). M3/38 produced an intense reaction product in the placenta, decidua basalis and metrial gland (Fig. 3c). Despite heavy reaction in these areas, very little was

detected on the embryonic side of Reichert's membrane (Fig. 4c). The reactivity that was detected on the embryonic side of Reichert's membrane appeared as a dried soluble product and is likely to have originated in the maternal side of the membrane as the section was being placed on the microscope slide, since it was not detected in other areas of the embryonic cavity. In contrast, M3/84 reactivity was distributed throughout the entire uterus and embryonic tissues (Fig. 3e). It was especially intense in the metrial gland and spongiotrophoblast region of the placenta. Although some M3/84 reactivity was present in

Fig. 4. Day 15 pregnant uterus (enlargement of small box in Fig. 3) stained with (a) M1/934, (b) media control, (c) M3/38, (d) 53-6.72 (immunoglobulin G2a isotype control), (e) M3/84, and (f) PC-61.5 (immunoglobulin G1 isotype control). P: placenta; R: Reichert's membrane; U: uterus; Y: yolk sac; A: amniotic cavity. Scale bar = 1 mm.

the mesometrial connective tissue it was in sharp contrast to the intense reaction within the uterus. In all sections, media and isotype-matched monoclonal antibody controls were negative or had distinctly different patterns of reactivity.

The M3/38-reactive components were further characterized by SDS-PAGE western blotting and found to have a molecular mass similar to the M3/38-reactive species (Mac-2) of macrophages (Fig. 5). M3/84 reactivity was not detected by western blot analysis.

Discussion

Results of the experiments reported here demonstrate that determinants recognized by the monoclonal antibodies M3/38 and M3/84 are expressed in distinctly different patterns during progressive gestational stages of the mouse uterus. This regional molecular specialization within the developing utero-placental unit indicates the existence of previously unsuspected compartmentalization that may have functional significance.

M3/38 was initially characterized by its ability to react with Mac-2, a 32 kDa macrophage cell surface glycoprotein (Ho and Springer, 1982). It is believed to be the major non-integrin laminin-binding protein of macrophages and may be involved in cell adhesion (Woo *et al.*, 1990). Sequencing studies indicate that Mac-2 is identical to carbohydrate-binding protein 35 (CBP35) (Jia and Wang, 1988; Cherayil *et al.*, 1989), tumour metastasis-associated galactoside-binding protein (L-34) (Raz *et al.*, 1989), and lung lectin (L-29) (Oda *et al.*, 1991). It is also highly homologous to a rat IgE-binding protein (Cherayil *et al.*, 1989). The presence of M3/38 immunoreactivity with PBS-soluble placental preparations is consistent with previous reports documenting the presence of Mac-2 (CPB-35/L-34/L-29/IgE-binding protein) in both the cytosol and the extracellular medium of cultured cells (Cherayil *et al.*, 1989; Rosenberg *et al.*, 1991). Expression of Mac-2 on the cell surface appears to be due to non-covalent association with the cell membrane, possibly through carbohydrate recognition sites. Woo *et al.* (1991) identified dimers of Mac-2 that may play a role in cell adhesion or migration by binding to oligosaccharide

Fig. 5. SDS-PAGE western blot analysis. P388D1 cells (lane 1) and soluble extracts of day 15 placenta (lane 2) and uterus (lane 3) were separated by SDS-PAGE and transferred to nitrocellulose. Replicate nitrocellulose sheets were incubated in (a) media or (b) M3/38 monoclonal supernatant followed by alkaline phosphatase-conjugated goat anti-rat immunoglobulin. Relative molecular mass is noted along the right margin.

moieties on both the cell surface and on matrix proteins such as laminin. The involvement of galactoside-binding lectins in cell migration and anchorage-independent growth regulation are further supported by the correlation of L-34 expression and metastatic potential of related tumour cell variants of the K-1735 melanoma and UV-2237 fibrosarcoma tumour systems (Raz *et al.*, 1986; Raz *et al.*, 1987).

M3/84 was originally described as reacting with a macrophage subpopulation-specific antigen, Mac-3 (Ho and Springer, 1983). This antibody has since been shown to react with a variety of tissues (Flotte *et al.*, 1983) and may be closely related, if not identical, to the lysosomal-associated membrane protein, LAMP-3 (Chen *et al.*, 1985). Although this study was not specifically designed to resolve subcellular organelles, immunoreactivity had a definite granular appearance in the cells and is consistent with previous studies that localize Mac-3 in the limiting lysosomal membranes. In day 1 uteri this granular pattern was primarily localized in the luminal side of the uterine epithelial cells.

M3/38 and M3/84 reactivity was not limited to cells expressing the common leucocyte antigen. On day 1, M3/84 was concentrated on the luminal side of the endometrium, an area that was negative for M1/934. On day 8, M1/934 positive leucocytes were distributed sparsely throughout most of the decidual mass but were heavily concentrated between the embryo and mesometrial side of the uterus. By comparison, M3/38 was restricted to cells immediately around the implanted blastocyst and M3/84 was positive throughout the entire decidual mass. Reactivity of these antibodies with non-leucocytic tissues is not a characteristic that is unique to

the uterus. Flotte *et al.* (1983) identified M3/38 and M3/84 reactivity in epithelial cells of kidney tubules, intestinal mucosa, bronchi, choroid plexus and epidermis. Cherayil *et al.* (1989) reported that the sequence of Mac-2 cDNA was identical to that of CBP35, found on fibroblasts. Chen *et al.* (1985) identified Mac-3 in NIH 3T3 and HaNIH cells. Molecular characteristics of the immunoreactive molecules from many of these tissues has not been thoroughly assessed. However, western blotting data from this study indicate that M3/38-reactive molecules from the uterus, placenta and P388D1 cells have molecular weights similar to those reported for Mac-2 (Ho and Springer, 1982; Cherayil *et al.*, 1989; Woo *et al.*, 1990).

This study establishes a direct relationship between the expression of M3/38 and M3/84 reactivity in the uterus and processes that occur during normal implantation and embryonic development. Although functions for these molecules have not been clearly defined, their stage-specific expression on pre-implantation blastocysts (Weitlauf and Knisley, 1992) and postimplantation fetal and uterine tissues suggest some degree of coordinated cellular function between maternal and fetal tissues. The pattern of M3/38 binding that occurs from implantation through midgestation is closely associated with areas where maternal and fetal tissues come into contact. Thus this molecule may play a role in controlling maternal recognition of the fetus or it may be involved in the transport of materials between the mother and fetus. The ubiquitous reaction of M3/84 with both fetal and uterine tissues may result from a soluble component that is synthesized by and released from one or more types of cell and bound by others throughout the maternal-fetal unit. Alternatively, direct synthesis by several different types of tissue may reflect common cellular functions, such as increased lysosomal activity, that are involved in growth and development of the uteroplacental unit. Continuing studies toward identification of the sites of synthesis and mechanisms that regulate expression of these molecules will further our understanding of the functional significance of microenvironmental compartments at the maternal-fetal interface.

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References

- Ceredig R, Lowenthal JW, Nabholz N and MacDonald HR (1985) Expression of interleukin-2 receptors as a differentiation marker on intrathymic stem cells *Nature* **314** 98–100
- Chen JW, Murphy TL, Willingham MC, Pastan I and August JT (1985) Identification of two lysosomal membrane glycoproteins *Journal of Cell Biology* **101** 85–95
- Cherayil BJ, Weiner SJ and Pillai S (1989) The Mac-2 antigen is a galactose-specific lectin that binds IgE *Journal of Experimental Medicine* **170** 1959–1972
- Finn CA and McLaren A (1967) A study of the early stages of implantation in mice *Journal of Reproduction and Fertility* **34** 247–253
- Flotte TJ, Springer TA and Thornbeck GJ (1983) Dendritic cells and macrophage staining by monoclonal antibodies in tissue sections and epidermal sheets *American Journal of Pathology* **111** 112–124
- Ho M-K and Springer TA (1982) MAC-2, a novel 32,000 Mr mouse subpopulation-specific antigen defined by monoclonal antibodies *Journal of Immunology* **128** 1221–1228

- Ho M-K and Springer TA** (1983) Tissue distribution, structural characterization, and biosynthesis of Mac-3, a macrophage surface glycoprotein exhibiting molecular weight heterogeneity *Journal of Biological Chemistry* **258** 636–642
- Jia S and Wang JL** (1988) Carbohydrate binding protein 35. Complementary DNA sequence reveals homology with proteins of the heterogeneous nuclear RNP *Journal of Biological Chemistry* **263** 6009–6011
- Laemmli UK** (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4 *Nature* **227** 680–685
- Ledbetter JA and Herzenberg LA** (1979) Xenogeneic monoclonal antibodies to mouse lymphoid differentiation antigens *Immunological Reviews* **47** 63–90
- Oda Y, Leffler H, Sakakura Y, Kasai K and Barondes SH** (1991) Human breast carcinoma cDNA encoding a galactoside-binding lectin homologous to Mac-2 antigen *Gene* **99** 279–283
- Parr EL, Tung HN and Parr MB** (1987) Apoptosis as the mode of uterine epithelial cell death during embryo implantation in mice and rats *Biology of Reproduction* **36** 211–225
- Raz A, Meromsky L and Lotan R** (1986) Differential expression of endogenous lectins on the surface of nontumorigenic, tumorigenic and metastatic cells *Cancer Research* **46** 3667–3672
- Raz A, Meromsky L, Zvibel I and Lotan R** (1987) Transformation-related changes in the expression of endogenous cell lectins *International Journal of Cancer* **39** 353–360
- Raz A, Pazerini G and Carmi P** (1989) Identification of the metastasis-associated, galactoside-binding lectin as a chimeric gene product with homology to an IgE-binding protein *Cancer Research* **49**, 3489–3493
- Rosenberg I, Cherayil BJ, Isselbacher KJ and Pillai S** (1991) Mac-2-binding glycoproteins *Journal of Biological Chemistry* **266** 18731–18736
- Springer TA** (1981) Monoclonal antibody analysis of complex biological systems *Journal of Biological Chemistry* **256** 3833–3839
- Springer TA, Galfe DS, Secher DS and Milstein C** (1978) Monoclonal xenogeneic antibodies to murine cell surface antigens: identification of novel leukocyte differentiation antigens *European Journal of Immunology* **8** 539–551
- Towbin H, Staehelin T and Gordon J** (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedures and some applications *Proceedings of the National Academy of Sciences, USA* **76** 4350–4354
- Weitlauf HM and Knisley KA** (1992) Changes in surface antigens on preimplantation mouse embryos *Biology of Reproduction* **46** 811–816
- Welch AO and Enders AC** (1987) Trophoblast–decidual cell interactions and establishment of maternal blood circulation in the parietal yolk sac placenta of the rat *Anatomical Record* **217** 203–219
- Welch AO and Enders AC** (1991) Chorioallantoic placenta formation in the rat: I. Luminal epithelial cell death and extracellular matrix modifications in the mesometrial region of implantation chambers *American Journal of Anatomy* **192** 215–231
- Woo H-J, Shaw LM, Messier JM and Mercurio AM** (1990) The major non-integrin laminin binding protein of macrophages is identical to carbohydrate binding protein 35 (Mac-2) *Journal of Biological Chemistry* **265** 7097–7099
- Woo H-J, Lotz MM, Jung JU and Mercurio AM** (1991) Carbohydrate-binding protein 35 (Mac-2), a laminin-binding lectin, forms functional dimers using cysteine 186 *Journal of Biological Chemistry* **266** 18419–18422