MRI Analysis of Angiogenesis During Mouse Embryo Implantation

Vicki Plaks,1 Vyacheslav Kalchenko,2 Nava Dekel,1 and Michal Neeman1*

Uterine receptivity and embryo implantation depend on local induction of angiogenesis and vascular permeability. Poor uterine receptivity has been implicated in implantation failure; however, relatively little is known about the mechanism that underlies endometrial vascular hyperpermeability in implantation sites. Here we show that contrast-enhanced (CE)-MRI and fluorescence microscopy using biotin-BSA-GdDTPA allowed high-resolution detection and quantitative assessment of mouse embryo implantation sites as early as embryonic day 4.5 (E4.5), and subsequent vascular expansion at E5.5. Vessel permeability, but not blood volume, was significantly elevated in E4.5 implantation sites relative to nonimplanted uterus, showing that elevation of vascular permeability is a very early response preceding E4.5. A significantly increased blood volume was detected by MRI and fluorescence microscopy in implantation sites between E4.5 and E5.5. On the other hand, despite the increase in blood volume, implantation sites showed only a small nonsignificant further increase in vascular permeability during these 2 days, demonstrating the rapid dynamics of vascular remodeling during the early days of pregnancy. Functional imaging by MRI, as reported here, allows multiparametric measurement of angiogenesis during normal mouse implantation and would facilitate the application of MRI to evaluate involvement of the vasculature in mouse models of impaired implantation. Magn Reson Med 55:1013–1022, 2006. © 2006 Wiley-Liss, Inc.

Key words: MRI; angiogenesis; embryo implantation; fluorescence microscopy; decidua

Implantation of mammalian embryos at the blastocyst stage into the uterine endometrium during the early stages of pregnancy involves a complex series of precisely synchronized physiological and cellular events, including significant vascular remodeling and angiogenesis (1). In this work we used the mouse as a model to study the early events of implantation, with the aim of developing a methodology to evaluate genetic aberrations that can lead to implantation disorders.

In brief, implantation in the mouse starts as blastocysts apposition at their sites of implantation in the endometrium and attach via the mural trophoderm layer (E4-4.5, vaginal plug = E0.5). Blastocyst attachment triggers the uterine stroma to rapidly proliferate and differentiate into a spongy mass of cells, known as decidua, which surround the blastocyst. Decidualization is primed by ovarian steroid hormones and is associated with induction of angiogenesis, including increased uterine vascular permeability and development of maternal vessels. Consequently, the maternal vessels dramatically increase in number and diameter in order to supply the fetus’s growing needs for oxygen and metabolites (2). The decidua stores glycogen and will sustain the embryo until the placenta and fetal circulation have developed (~E10). Implantation continues as the epithelium, which separates the blastocyst from the stroma, is eroded and fetal trophoblast cells invade the decidua and the inner myometrium to reach maternal vessels. Concurrently, these specialized epithelial cells develop endothelial-like characteristics, which allows them to replace the maternal endothelium and divert maternal blood to the placenta (3).

Embryo implantation and development are critically dependent on the spatial and temporal regulation of angiogenesis and localized uterine vascular permeability. Embryo implantation failure is clinically relevant to recurrent pregnancy loss and low success of in vitro fertilization programs in achieving pregnancy. Many human conceptuses fail to implant because of genetic or metabolic abnormalities (4,5), but poor uterine receptivity characterized by impaired uterine hyperpermeability has been widely proposed as another cause of implantation failure (6). Several complications of pregnancy, such as pre-eclampsia and intrauterine growth restriction, have been attributed to deficient trophoblast invasion of the placental bed spiral arterioles (7). Similarly, excessive invasion of the uterine wall can also severely compromise pregnancy. Therefore, it is important to characterize the processes that control uterine angiogenesis and subsequent placental formation.

The low-molecular-weight MR contrast material Gd-DTPA was previously used to detect implantation sites 10 hr post artificially-induced decidualization in rats (8). GdDTPA was further used to visualize implantation sites on the night of day 5 of pregnancy in the rat (equivalent to E4-4.5 in the mouse) after surgical alignment of the uteri (9). These early studies demonstrated the ability of MRI to visualize the first stages of pregnancy in rodents.

In the study reported here we evaluated the use of macromolecular contrast-enhanced (CE)-MRI for quantitative, high-resolution, noninvasive, and dynamic analysis of embryo implantation in mice. The use of mice instead of rats is challenging because mice have a 10-fold lower body weight relative to rats, but it would open the possibility for analysis of transgenic and knockout models. Furthermore, to allow analysis of pregnancy progression and outcome, the studies reported here included no surgical intervention, as was done previously for imaging of implantation of embryos in the rat (9). We previously reported that Gd-
DTPA conjugated to bovine serum albumin and biotin (biotin-BSA-GdDTPA) allows noninvasive MRI, as well as fluorescence microscopy validation of vascular development and associated hyperpermeability in tumors (10), ischemic injury (11), and ovarian xenografts (12). Here we show that biotin-BSA-GdDTPA, because of its selective extravasation from hyperpermeable vessels and its slow diffusion and clearance from the extracellular space, allowed high-resolution detection of embryo implantation sites in mice as early as at E4.5, and subsequent vascular expansion at E5.5, and enabled quantitative assessment of embryo implantation and vascular remodeling. The use of dual-modality contrast material enabled histological validation of the CE-MRI data. Thus, macromolecular CE-MRI of fetal implantation can be used to assess implantation disorders in transgenic and knockout mice.

MATERIALS AND METHODS

Animal Model of Embryo Implantation

Female ICR mice (12 weeks old) were analyzed on the fifth (E4.5) and sixth (E5.5) days of pregnancy (vaginal plug = E0.5, indicated mating had occurred). All of the animal experiments were approved by the Weizmann Institutional Animal Care and Use Committee.

In Vivo CE-MRI Studies

MRI experiments were performed on a horizontal 4.7 T Bruker Biospec spectrometer (Karlsruhe, Germany) using a whole-body birdcage RF coil, with a diameter of 7.5 cm. No quadrature detection was applied.

The mice were anesthetized with intraperitoneal injection of 75 mg/kg Ketaset (ketamine; Fort Dodge Laboratories, Fort Dodge, IA, USA) and 3 mg/kg XYL-M 2% (xylose; VMD, Arendonk, Belgium) followed by the subcutaneous addition of about 30% of the initial dose to prolong anesthesia. Anesthetized mice were placed prone on a polymethylmethacrylate (Perspex) board, immobilized using adhesive tape, and covered with a paper blanket to reduce the temperature drop during the measurement.

A bovine serum albumin (BSA)-based macromolecular contrast material, biotin-BSA-GdDTPA (biotin$_3$-BSA-GdDTPA$_{33}$; about 82 kDa), was prepared as reported previously (10,13) and injected through a tail vein catheter as bolus (18 mg/mouse in 0.2 ml of PBS).

At the end of the MRI session, Evans blue (Sigma, St. Louis, MO, USA; 1% w/v in saline, 100 μl (14)) was i.v. injected via the tail vein and allowed to circulate for 10 min to enable ex vivo detection of implantation sites. Evans blue binds serum proteins and accumulates in the interstitial tissue around the implanting embryos (8) in a similar manner to the biotin-BSA-GdDTPA.

Before the animals were killed, BSA labeled with rhodamine (BSA-ROX) and prepared as reported previously (13) was injected via the same tail vein catheter for the detection of functional blood vessels. The animals were euthanized 1–3 min after BSA-ROX administration by anesthesia overdose, and the uteri were retrieved for histological analysis.

MRI Data Acquisition

A series of variable-flip-angle precontrast $T_1$-weighted 3D gradient-echo (3D-GE) images were acquired to determine the endogenous precontrast $R_1$ (pulse flip angle = 15°, 5°, 30°, 50°, 70°; repetition time (TR) = 10 ms; time to echo (TE) = 3.6 ms; two averages; spectral width = 50000 Hz; FOV = 5 × 5 × 5 cm; matrix = 128 × 128 × 64, zero-filled to 128 × 128 × 128 resulting in a voxel resolution of 0.39 × 0.39 × 0.39 mm (0.78 mm is the resolution in the third dimension before zero-filling); total acquisition time per frame = 164 seconds; frequency-encoding direction: head–foot).

For dynamic postcontrast imaging, $T_1$-weighted 3D-GE images were acquired from the time of biotin-BSA-GdDTPA administration and up to ~30 min. The first image was acquired 0–164 s after administration of the contrast material, with signal intensity dominated by the time of acquisition of the center of k-space at 82 s (pulse flip angle = 15°, and all other parameters as stated above).

MRI Data Analysis

The MRI data were analyzed on an Indigo-2 workstation (Silicon Graphics Inc., Mountain View, CA, USA) using Paravision (Bruker, Rheinstetten, Germany) and Matlab (The Math Works Inc., Natick, MA, USA) software.

Precontrast $R_1$ values ($R_1$ pre; $R_1$ = 1/$T_1$) were determined for each selected region of interest (ROI) using the 3D-GE data sets:

$$I = \frac{M_s \sin(\alpha - e^{-T_1 R_1 \text{pre}})}{1 - \cos \alpha e^{-T_1 R_1 \text{pre}}} \quad [1]$$

Where $I$ is the signal intensity as a function of the pulse flip angle $\alpha$, and the pre-exponent term, $M_s$, includes the spin density and the $T_1$ relaxation, which are assumed to be constant in this experiment. The mean noise (signal intensity measured with flip angle $\alpha = 0$) was subtracted from precontrast signal intensity.

Postcontrast $R_1$ values ($R_1$ post) were calculated from pre- and postcontrast 3D-GE mean signal intensities:

$$I_{\text{pre}} = \frac{M_s \sin(\alpha - e^{-T_1 R_1 \text{pre}})}{1 - \cos \alpha e^{-T_1 R_1 \text{pre}}} \quad I_{\text{post}} = \frac{M_s \sin(\alpha - e^{-T_1 R_1 \text{post}})}{1 - \cos \alpha e^{-T_1 R_1 \text{post}}} \quad [2]$$

where $R_1$ post is the calculated value obtained by Eq. [1] from selected ROIs in the appropriate organ, the flip angle is 15°, and TR is 10 ms. Breathing and bowel motion precluded the generation of parameter maps using voxel-by-voxel fitting as reported previously (13,15). Thus, ROIs at implantation sites were selected manually from relevant slices of the 3D data sets. The shape of the ROI for each implantation site was depicted on the scan of 7.5 min post contrast administration, and its position at each time point was corrected manually within the 3D data set to account for implantation site movement. Corrections were on the order of 0.5–1 mm.

Finally, concentrations were calculated based on the relaxivity of biotin-BSA-GdDTPA measured at 4.7 T ($R = 186.0$ mM$^{-1}$ s$^{-1}$, which was determined as described previously (10)):
\[ Ct = \frac{[\text{biotin} - \text{BSA} - \text{GdDTPA}]}{R_{\text{pred}} - R_{\text{post}}} \]  

The concentration values of the contrast material were calculated for each selected implantation site for 10 time points after administration of the contrast material (Ct; up to ~30 min postcontrast).

For each postcontrast time point the biotin-BSA-GdDTPA concentration in the ROI of the implantation sites was divided by the concentration of biotin-BSA-GdDTPA in blood \( C_{\text{blood}} \), calculated in ROI depicting the vena cava, which was included in the image data set, and extrapolated to time 0. Linear regression of these temporal changes in \( Ct/C_{\text{blood}} \) yielded two parameters (the blood volume fraction (fBV) and permeability surface area product (PS)) that characterize vascular development:

\[ \frac{Ct}{C_{\text{blood}}} = (fBV + PS\cdot t) \]  

fBV (\( C_t/C_{\text{blood}} \)) was derived from the ratio between the concentration of biotin-BSA-GdDTPA in the tissue (Ct) extrapolated to the time of administration of the contrast material (Ct) and its concentration in the vena cava extrapolated to the time of administration of the contrast material (Ct).

PS (\( (C_t - C_{\text{end}})/(C_{\text{blood}}\cdot t!)) \) is the rate of contrast extravasation and accumulation in the tissue derived from the slope of the linear regression of the first 30 min post contrast material administration (t = 30).

The mean changes in PS and fBV for all mice on the relevant embryonic day (E4.5 or E5.5) were calculated from the linear regression, and were also recalculated from mean PS and fBV values of all implantation sites per mouse. Both methods of calculation yielded similar results. The mean values \( \pm SE \) are reported.

Histology, Immunohistochemistry, and Fluorescence Microscopy

Samples of implanted uteri were fixed, sectioned serially at 4 \( \mu \)m thickness, and stained for eosin and hematoxylin, pericytes, and vascular smooth muscle cells (using anti alpha-smooth muscle actin (SMA); Sigma-Aldrich, Rehovot, Israel), endothelial cells (using GSL-1 lectin, from \textit{Griffonia simplicifolia}; Sigma-Aldrich, Rehovot, Israel), and biotin-BSA-GdDTPA (using fluorescein-labeled avidin, avidin-FTTC; Molecular Probes, San Francisco, CA, USA) as previously described (15).

Fluorescence Image Analysis

Analysis was performed on paraffin sections stained with avidin-FTTC for detection of the biotinylated MR contrast material. All fluorescent images were acquired under identical conditions and analyzed using ImageJ software (Wayne Rasband, NIH, USA). Vessel density was determined by calculating the percent area of vessels per implantation site area. Based on areas of high fluorescence intensity due to contrast material within well-resolved blood vessels, a mask was generated to determine the vessel area. The high fluorescent intensity was sampled mostly from myometrial vessels or decidual vessels at early time points (5 and 10 min) because their contours can be clearly discriminated from the interstitial space. A nonlinear intensity correction was made by applying a gamma filter of ImageJ with a gamma value of 5. Using this filter, medium-intensity objects can be made fainter without dimming the bright objects. An automatic threshold based on the entropy of the histogram was then applied (ImageJ plugin) to finalize the determination of vessel area. Regions of very high permeability could show overestimation of vessel area, but the large difference in fluorescence intensity between resolved vessels and the interstitial space implied that this bias is lower than 5%. An average of three discrete ROIs inside an implantation site were used to determine the fraction of vessel area. Permeability was calculated from the ratio between the signal intensity outside and inside vessels (12). In brief, a mask was used to determine vessel area, as described above. The vessel area was excluded from the selected ROI in order to determine the area located outside the vessels. Fluorescent intensity outside the vessel area mask (i.e., decidua/stroma) was then measured. An average of three discrete ROIs inside an implantation site was used to determine the signal intensity coefficient (\( I_{\text{stroma}}/I_{\text{vessel}} \)) per each time postcontrast. The same approach was applied to calculate permeability in ROIs from the myometrium and nonimplanted uterine sites.

Statistical Analysis

Vessel Density

Using the Shapiro-Wilk test for normality, both the MRI and fluorescent data were assumed to be normally distributed. Therefore, a two-sample, two-tailed \( t \)-test was applied to the fluorescent data, and then a two-sample, one-tailed \( t \)-test was applied to the MRI data under the assumption that vessel density increases as the implantation process progresses.

Permeability

The MRI data were not normally distributed according to the Shapiro-Wilk test for normality (\( P = 0.0032 \)); therefore, the Wilcoxon-rank test was applied. The fluorescent data were analyzed with two-sample, two-tailed \( t \)-test to check individual time points postcontrast between the 2 days (E4.5 and E5.5). For consolidation, a comparison of the regression line slopes for each of the 2 days was also employed.

RESULTS

MRI Census of Mouse Embryo Implantation Sites at E4.5 and E5.5

Signal enhancement post i.v. injection of biotin-BSA-GdDTPA allowed detection of implantation sites in maximal intensity projections (MIPs) derived from 3D-GE MRI data sets at both E4.5 and E5.5 (Fig. 1a and b, respectively). The number of implantation sites detected by ex vivo examination post i.v. injection of Evans blue (EB, Fig. 1c and d) was found to correlate well with the number of implantation sites counted from in vivo MRI-derived MIPs in the
same animal (Fig. 1e, N = 11 mice, R² = 0.98). Note the absence of false positives (the 0.0 point in the graph, Fig. 1e; N = 5) in spontaneous nonpregnant uteri (animals with detected vaginal plugs that exhibited no pregnancy in ex vivo examination). Measurement of the implantation site diameter based on in vivo MRI MIPs (as in Fig. 1a and b) revealed that the diameter increased by 1.75-fold from E4.5 to E5.5 (diameterE4.5 = 1.34 ± 0.11 mm (N = 9 implantation sites in three dams), diameterE5.5 = 2.35 ± 0.18 mm (N = 9 implantation sites in three dams), P = 0.004) in agreement with the increased diameter measured ex vivo after EB i.v. injection (as in Fig. 1c and d), as well as with previously reported data (16–18).

The ability of the embryos to develop to term post i.v. injection of BSA-GdDTPA during implantation was explored. Two animals that were scanned on E4.5 were left to reach parturition when a normal number of healthy-looking pups were born (9 and 12 pups in each dam respectively). The pups were followed for 2 weeks and appeared to be developing normally. Therefore, MRI analysis using biotin-BSA-GdDTPA conducted during the early stages of embryo implantation did not lead to termination of pregnancy or absorption of embryos, and did not result in any gross abnormalities in embryonic development.

Implantation Sites Show Elevated MRI-Derived Angiogenic Parameters (fBV and PS)

The changes in MRI signal enhancement measured during a follow-up period of 30 min were used to derive the concentrations of biotin-BSA-GdDTPA that extravasated and accumulated in the implantation sites. The biotin-BSA-GdDTPA concentrations at each time point, normalized by the concentration in the blood, were used to derive the fBV and vascular PS (Fig. 2a; E4.5, N = 6 mice, total of 13 implantations sites; E5.5, N = 8 mice, total of 14 implantation sites). The PS values (Fig. 2c) were found to be significantly elevated in implantation sites and were comparable to other models of angiogenesis (12). With MRI we noninvasively detected a significant increase in blood volume (fBV) between E4.5 and E5.5 (P = 0.04). Vascular PS was significantly elevated at E4.5 relative to the nonimplanted uteri (which were unenhanced), and showed only a slight nonsignificant increase between E4.5 and E5.5 (P = 0.28). Thus, MRI monitoring of the early events of vascular remodeling associated with angiogenesis in mouse embryo implantation sites showed an early increase in permeability prior to E4.5, followed by an increase in blood volume between E4.5 and E5.5.

Localization of Biotin-BSA-GdDTPA Correlates With MR Signal Enhancement in Implantation Sites

The localization of biotin-BSA-GdDTPA in histological cross sections of E4.5 implantation sites was visualized using avidin-FITC stain (Fig. 3a and b). At 30 min after i.v. administration, extravasated biotin-BSA-GdDTPA (stained with avidin-FITC; green) was detected in the decidua with a gradient of decreasing intensity toward the embryo. No contrast material was found to penetrate the embryo or the myometrium. BSA-ROX (red) injected 1–3 min before the animals were euthanized assisted with the visualization of functional blood vessels (Fig. 3b, a magnified region of the region marked in Fig. 3a). Note that the early injected biotin-BSA-GdDTPA colocalized with the later injected BSA-ROX (yellow) in blood vessels (arrows). MRI revealed an unenhanced area in the center of the implantation site that was consistent with the absence of contrast material around the embryo (Fig. 3c and magnified region in d; arrows; note also the similar MRI enhancement pattern for all implantation sites in Fig. 1a and b). Thus, the MRI-detected signal enhancement of the rim of the implantation sites (Fig. 3c and d) was consistent with the histolog-
localization of extravasated biotin-BSA-GdDTPA (Fig. 3a and b).

Monitoring Angiogenesis in Embryo Implantation Sites by Immunohistochemistry-Kinetics of Biotin-BSA-GdDTPA Extravasation Is Consistent With the MRI Data

To validate the changes in blood volume and permeability detected by MRI (Fig. 2), histological cross sections of E4.5 and E5.5 (Fig. 4) of implantation sites retrieved at 5, 10, 22, and 30 min after i.v. injection of biotin-BSA-GdDTPA (green) were examined. Vessel density was determined by calculating the percent of intravascular area in the implantation site (Fig. 5). Consistent with the MRI data, vessel density significantly increased between E4.5 and E5.5 (E4.5: N = 5 mice, total of seven implantation sites; E5.5: N = 4 mice, total of five implantation sites; P = 1.7 × 10^{-4}; Fig. 5a). The change in vessel density is readily evident in images of E4.5 and E5.5 implantation sites at 5 and 10 min postcontrast (Fig. 4a and b vs. e and f), as the biotin-BSA-GdDTPA is still mostly localized within the capillaries (E4.5 capillaries look smaller and less extended than E5.5 capillaries). Permeability was determined from the ratio between fluorescence intensity in the interstitial space outside the vessels and the intensity inside the vessels for indicated time points (up to 30 min) at E4.5 and E5.5 (signal intensity coefficient; three measurements each, Fig. 5c). At each time point the signal intensity coefficient was higher for E5.5 compared to E4.5 implantation sites, which indicated increased permeability at E5.5 compared to E4.5 implantation sites. However, only 5 and 22 min after contrast time points exhibited a significant difference in the signal intensity coefficient between E4.5 and E5.5 (P = 0.03 and P = 5.07 × 10^{-8}, respectively). More importantly, no significant difference was found between linear regression of all time points (5, 10, 22, and 30 min) for E4.5 (0.005 ± 0.002, R² = 0.82) and E5.5 (0.006 ± 0.003, R² = 0.65). Thus, similarly to the MRI data, the overall increase in permeability between E4.5 and E5.5 was not statistically significant. These results, in agreement with the MRI data, point to increased permeability that occurred prior to E4.5 followed by increased vessel density that occurred between E4.5 and E5.5.

Extravasation of Biotin-BSA-GdDTPA Is Confined to Implantation Sites

The selective enhancement of embryo implantation sites vs. nonimplanted uterine sites exhibited in the MR images was also manifested in the analysis of histological cross sections of the uteri (Figs. 5 and 6). Vessel density in the nonimplanted uterine sites (non-imp) or myometrium (myo) of E4.5 and E5.5 uteri (Fig. 5b) was not significantly different from that in E4.5 implantation sites (P = 0.12, eight mice and total of eight non-imp or myo) but significantly lower than vessel density measured in E5.5 implantation sites (P = 6.54 × 10^{-8}, seven mice and total of seven non-imp or myo). Permeability in the non-imp and myo at 30 min after i.v. injection of contrast material (Fig. 5d) was significantly lower compared to that detected in implantation sites at the corresponding time point after administration of the contrast material (Fig. 5c; P = 0.0005 for myo, P = 0.0009 for nonimplanted uterus in comparison with implantation-site values of E4.5 and E5.5 taken together). This was also correct for all other time points examined (data not shown). Biotin-BSA-GdDTPA extravasated from capillaries was located in the stroma (that started to convert into the decidua) of E4.5 implantation sites (Fig. 6a and b). In contrast, the biotinylated contrast material was found to be restricted to the capillaries located in a nonimplanted uterine site in the same mouse (Fig. 6c and d). Moreover, biotin-BSA-GdDTPA was still localized in the implantation site 2.5 hr after i.v. injection, but was not detected in nonimplanted uterine sites located...
away from the implantation sites (data not shown). Therefore, the use of macromolecular MR contrast material, which selectively extravasates from hyperpermeable vessels, allows differentiation between highly-enhanced implantation sites and poorly-enhanced nonimplanted uteri.

Vascular Development in E4.5 and E5.5 Implantation Sites

The cascade of vascular remodeling characterized by CE-MRI of implantation sites was consistent with the structural properties of the neovasculature at E4.5 and E5.5. Lectin immunostaining of endothelial cells of E4.5 (Fig. 7a) and E5.5 (Fig. 7b) implantation sites indicated extensive infiltration of endothelial cells, which was more apparent in E5.5 than in E4.5. This angiogenic response can account for the increase in vessel density from E4.5 to E5.5, and also for the elevated permeability (Figs. 2 and 4). Capillaries with lumen were mostly detected at a distance of ~0.2 mm from the embryo, consistent with the finding that endothelial cells proliferating in the inner regions of the decidua had not yet established functional capillaries. The unenhanced center of the implantation site as revealed by MRI and fluorescence microscopy (Fig. 3) is a result of this absence of functional capillaries in the decidua proximate to the embryo. Alpha-SMA immunostaining of E4.5 (Fig. 7c) and E5.5 (Fig. 7d and e) implantation sites indicates smooth muscle in the myometrium and smooth muscle coated mature, low-permeable blood vessels in the myometrium and its vicinity as well as in nonimplanted uterine sites (Fig. 7f and g). Thus, the distribution of immature vessels (stained only with lectin) in the decidua correlates spatially with extensive vascular permeability of the implantation site capillaries and marked interstitial accumulation of biotin-BSA-GdDTPA within the implantation site (Figs. 3 and 4).

**DISCUSSION**

This work demonstrates the feasibility and validity of macromolecular CE-MRI for detecting mouse decidualization and embryo implantation, and characterizing the associated induction of maternal angiogenesis. With the use of this MRI methodology, pregnancy in the mice was detected as early as E4.5, immediately postimplantation. Furthermore, the number of implantation sites detected by MRI matched with the actual number of implantation sites detected ex vivo after intravenous injection of Evans blue. The ability to noninvasively examine early implantation in intact animals has important implications for providing information that was not previously accessible by conventional approaches. Traditional methods of examination, such as histology, cannot follow dynamic processes associated with changes in blood flow and vascular permeability, such as vascular development. Although vascular hyperpermeability in the endometrium is known to be essential for the progression of later events during pregnancy (19), relatively little is known about the mechanism by which this occurs. In this study macromolecular CE-MRI proved to be sensitive for measuring differences in vascular development of embryo implantation sites during 2 consecutive days of early pregnancy in mice.

In this work MRI revealed substantial vascular hyperpermeability that occurred very early during implantation, prior to E4.5, and was followed by slight nonsignificant increase in permeability between E4.5 and E5.5. On the other hand, blood volume did not change prior to E4.5, but showed a significant increase between E4.5 and E5.5, demonstrating the rapid dynamics of vascular remodeling during the early days of pregnancy. The MRI results were validated by fluorescence microscopy, which revealed the localization of the MR contrast material in the implanta-
tion sites without penetrating the embryo, as well as the decidua in its vicinity and the myometrium or nonimplanted uterine sites. The lack of functional capillaries in the decidua proximate to the embryo was consistent with the unenhanced center of the implantation sites in the MR images. Thus, functional immature hyperpermeable capillaries in the secondary decidual zone characterized embryo implantation and enabled the detection of implantation sites by biotin-BSA-GdDTPA-enhanced MRI. Fluorescent microscopy of the MR contrast material also revealed a significant increase in permeability in the implantation sites relative to the myometrium and nonimplanted uteri. The blood volume increase (relative to the myometrium and nonimplanted uteri) was delayed and was significant only at E5.5. Moreover, uterine signal enhancement was restricted to the implantation sites with no enhancement in nonimplanted uteri. Therefore, signal enhancement in implantation sites on E4.5 was primarily a consequence of vascular permeability, while the vessel density increase contributed to the signal enhancement only from E5.5.

The pattern of expression of angiogenic factors in the implantation site was previously reported (2,20). Vascular development during embryo implantation is mainly governed by vascular endothelial growth factor (VEGF) and angiopoietins. VEGF expression is stimulated by hypoxia, cytokines (as interleukin-1 (21)), and various hormones (particularly estrogen (22)). VEGF promotes angiogenesis by acting directly on endothelial cells (19) and is one of the primary inducers of vascular permeability and angiogenesis in the uterus. In the mouse there is a shift from uniform expression of VEGF transcripts throughout the uterus at estrus and the first 4 days of pregnancy to an endometrial epithelial localization just before and during implantation. VEGFR-2 (Flk-1/KDR), a major transducer of VEGF signals, was expressed in blood vessels in the implantation sites on E4.5. With the differentiation of the stroma on E5.5, an

FIG. 4. Monitoring angiogenesis in embryo implantation sites by fluorescence microscopy. Cross sections of E4.5 (a–d) and E5.5 (e–h) implantation sites at 5, 10, 22, and 30 min after i.v. injection of biotin-BSA-GdDTPA as indicated. Biotin-BSA-GdDTPA was visualized in histological cross sections using avidin-FITC (green). All images were photographed with identical exposures. * Embryo. Scale bar: 100 μm.
increased number of Flk-1 blood vessels were present in the secondary decidual zone, but the primary decidual zone was devoid of such blood vessels (19).

Angiopoietins coordinate with VEGF to fully complement angiogenesis. Angiopoietin-1 (Ang-1) promotes angiogenic remodeling, including vessel maturation, stabilization (23), while Angiopoietin-2 (Ang-2) acts as an antagonist. With the onset of implantation, expression of Ang-1 and -2 was shown to be very low (2). On E5.5, Ang-1 was restricted to the secondary decidual cells and undifferentiated stromal cells away from the embryo, while Ang-2 was observed primarily in decidual cells close to the implanting embryo. The expression pattern of Tie-2, a receptor for the angiopoietins, was similar to that of Flk-1. Thus, Ang-2 collaborates with VEGF in front of invading vascular sprouts by blocking Ang-1-induced stabilization and maturation, while maintaining vascular plasticity and responsiveness to VEGF. In the absence of VEGF, Ang-2 antagonizes Ang-1 signaling and thus contributes to vessel regression (2).

Vessel regression in the inner regions of the decidua located near the embryo may explain the lack of functional capillaries and MR enhancement observed in the present study. Moreover, in accordance with the low permeability of the myometrium described here, the myometrium was shown to be negative for VEGF expression (20). Thus, functional permeable capillaries located in the secondary decidual zone give rise to the MR signal in the outer rim of the implantation site.

The ability to detect implantation sites by MRI was previously demonstrated with the use of GdDTPA (8,9). In this study, the use of macromolecular MR contrast material yielded improved specificity for detecting the implantation sites by reducing background vascular leak from

**FIG. 5.** Quantification of vessel density and vascular permeability from fluorescent labeling of biotin-BSA-GdDTPA. a: In correlation with the MR data, vessel density (percentage of vessels at the implantation site) increased significantly from E4.5 to E5.5. b: Vessel density in the myometrium (myo) and the nonimplanted uterine sites (non-imp) was not significantly different from that in the implantation sites in a on E4.5, but was significantly lower than in the implantation sites in a on E5.5 (i indicates significant difference, $P = 6.54 \times 10^{-6}$). c: Permeability (signal intensity coefficient calculated for indicated time points up to 30 min after contrast material i.v. injection) in implantation sites also increased from E4.5 to E5.5, but the overall data were not significant. d: Permeability (signal intensity coefficient) in myo and non-imp at 30 min post contrast was low compared to the same time point in c (ii indicates a significant difference between overall values measured at 30 min, $P \leq 0.0005$). * $P < 0.05$, ** $P < 0.0005$.

**FIG. 6.** Specific elevated extravasation of biotin-BSA-GdDTPA in implantation sites. a and b: Cross sections of an E4.5 implantation site. c and d: Cross sections of a nonimplanted uterine site in the same mouse. a and c: Fluorescence microscopy of avidin-FITC-stained sections. Tissue was retrieved 30 min after administration of biotin-BSA-GdDTPA. b and d: The same sections as in a and c, respectively, costained with hematoxylin. Note that the biotinylated contrast material (green) extravasated from blood vessels in the implantation site (a) but was restricted to blood vessels in the nonimplanted uterine site (c). e, embryo; ue, uterine epithelium; dec, decidua; st, stroma; myo, myometrium. Scale bar: 100 μm.
nonimplanted uterine sites. Previous studies mostly utilized i.v. injection of radioactive blood markers as $^{125}$I-labeled albumin (24) or $^{51}$Cr-EDTA (8,25) to quantify vascular development from uterine tissue retrieved for ex vivo examination. In the present study MRI enabled in vivo quantification of vascular development and allowed fine changes in these parameters to be determined over time during the duration of the decidualization process. Thus, macromolecular CE-MRI provides an opportunity to non-invasively characterize different animal models of impaired vascular development associated with decidualization and implantation.

In agreement with the high vascular permeability detected by MRI immediately postimplantation in the mouse, previous studies demonstrated an increase in vascular permeability to $^{125}$I-labeled human serum albumin on the evening of day 5 and morning of day 6 of pregnancy in rat implantation sites (equivalent to E4-4.5 in the mouse) (24). However, estimates of endometrial vascular permeability as assessed after i.v. injection of $^{125}$I-labeled albumin indicated a subsequent fall in this parameter between 9 and 27 hr post artificial decidualization (25,26), which is equivalent to the time point of E4.5 to E5.5 in the mouse. Along this line, although in this study MRI-detected vascular hyperpermeability characterizing the onset of embryo implantation (E4.5) still increased, it also slowed down with the progression of implantation (E5.5). This is exemplified by the nonsignificant difference in permeability between E4.5 and E5.5. In contrast to permeability characteristics, vascular development toward the end of the implantation process is characterized by a significant increase in blood volume compared to the time of initiation of implantation. Fluorescence microscopy and MRI revealed that the onset of angiogenesis in the implantation site was characterized by increased permeability. Vessel density increased later, while permeability remained elevated but did not increase further. This dissociation between vascular permeability and vascular development revealed by MRI and supported by fluorescent microscopy of biotin-BSA-GdDTPA implies that angiogenesis during embryo implantation is restricted and tightly regulated. Notably, vascular permeability is further attenuated during subsequent placentation, leading to exclusion of contrast material from the embryo (27,28).

In summary, we have demonstrated the ability to non-invasively characterize the dynamics of angiogenesis during the critical stage of embryo implantation without compromising pregnancy progression and embryonic development. The use of macromolecular CE-MRI at high field provided increased selectivity and improved resolution for detecting implantation sites. MRI can be used to detect pregnancy at very early time points and to follow this dynamic process in vivo in the same animals. With the establishment of angiogenesis parameters for normal implantation, MRI can now be applied to study mouse models of impaired implantation associated with angiogenic disorders.
ACKNOWLEDGMENTS

The authors thank Dr. Tamara Berkutzky for her help with preparation and analysis of histological sections, Dr. Nava Nevo for her help with tissue isolation, Dr. Edna Shechtman for her help with the statistical analyses, and Dr. Hagit Dafni and Dr. Tomer Israel for helpful discussions and criticism. This research was supported by a grant from the Israel Science Foundation and the Y. Leon Benoziyo Institute for Molecular Medicine at the Weizmann Institute of Science (to M.N.). N.D. is the current holder of the Kluznick Professional Chair.

REFERENCES