Novel noninvasive detection method for endometriosis: research and development of scintigraphic survey on endometrial implants in rats

In this experimental study on endometriosis, the majority of the implants were successfully detected with technetium- (^{99m}Tc) labeled red blood cell scintigraphy. (Fertil Steril® 2008;90:209–13. ©2008 by American Society for Reproductive Medicine.)

Endometriosis is defined as the presence of endometrial tissue outside the uterus. It is clinically associated with pelvic pain and infertility (1). Clinical and basic research on endometriosis has been limited, partly because of the lack of accurate noninvasive diagnostic techniques. The diagnosis of endometriosis generally is performed by visual inspection of the pelvis during laparoscopy (2). The invasiveness and associated morbidity of the laparoscopic procedure preclude its use for monitoring recurrences and response to therapy. Radiological techniques, however, have been used with varying degrees of success. Of them, ultrasonography is helpful only for ovarian endometriosis (3). Magnetic resonance imaging is more accurate in depicting soft tissue changes and has been useful for diagnosing several disorders of the female pelvis (4). However, it is not effective in detecting extraovarian endometrial adhesions and intraperitoneal implants (5). Endometrial and serum markers are another line of research with promising results for diagnosis (3, 6). However, they do not appear to be helpful in monitoring the progression of the disease.

To overcome the limitations of the techniques mentioned in the previous paragraph, novel methods have been used. In the method known as radioimmunodetection, radiolabeled antibodies, a class of imaging agents, are used to detect a target disease (3, 7, 8). The subject is scanned after these tracers are injected, to detect any radioactive accumulation. Radioactive labeling of a proper antibody against endometriotic tissue potentially may be helpful in detecting endometriosis and in assessing its extent. However, this technique was only studied as a clinical observation in a very few case reports and has never been tested experimentally (9, 10).

The present study was planned to investigate the role of scintigraphy in the noninvasive diagnosis of experimentally induced endometriosis in a controlled laboratory setting. Implants were labeled by using radioactive tracers that were targeted to them.

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MATERIALS AND METHODS

Twenty-four female Wistar rats (Inonu University Medical Faculty Animal Research Laboratory, Malatya, Turkey) that were 6–8 weeks of age and weighed 150–200 g were kept in temperature- (21–22°C) and humidity- (60% ± 5%) controlled conditions. A 12:12-hour light–dark cycle was maintained. Food and water were available ad libitum.

Endometriosis was induced surgically by using the method described by Vernon and Wilson (11). Implants were fastened onto the peritoneum only on the right side of the ventral abdominal wall, between inferior renal pole and bladder, close to an artery. The left side was not implanted and served as the control. Three weeks after the initial surgery, midventral laparotomy was performed to determine the attachment and viability of endometrial implants. Also, classification of the implants was performed as described by Ingelmo et al. (12). Briefly, vesicles at the suture sites were classified as grade 1 through grade 4 (grade 1, no vesicle; grade 2, vesicle $\leq$2 mm; grade 3, vesicle $\geq$2 mm but <4.5 mm; and grade 4, vesicle $\geq$4.5 mm).

The implanted rats developed a vesicle in 87.5% of their sutures. Of these, 80.9% had a diameter of >2 mm (grade 3 or 4). Of the 24 experimental rats, only 3 did not develop a vesicle at any suture. Three rats were excluded from the study because implants were nonviable in two of them and were covered by omentum in the other. The remaining rats with endometriosis were divided into two groups, as follows: 15 rats were injected IV through the tail vein with ^{99m}Tc-labeled red blood cell (^{99m}Tc-RBC), and 6 rats were injected with ^{131}I-labeled tamoxifen (^{131}I-Tx).

One week after the second laparotomy, scintigraphy was performed. Radioactive ^{131}I-Tx was prepared according to a study published elsewhere (13). We injected 0.3 mL (3.7 MBq) of that tracer into the tail vein. For the

^{99m}Tc-labeled RBC study, 50 μg of stannous chloride (Amerscan Stannous Agent; Amersham International, Buckinghamshire, UK) was prepared in 0.2 mL of saline and was injected into the tail vein. At the 30th minute, 0.2 mL (15 MBq) of ^{99m}Tc-pertechnetate (MON-TEK, Monrol Inc, Istanbul, Turkey) was injected into the tail vein.

All injections were applied with 26-G syringes. The rats were immobilized in a mold and placed in front of the gamma camera (ADAC; Vertex V60, Milpitas, CA). External images were obtained at 30 and 240 minutes after initial injection. Results were given in terms of the lesion-to-background ratio of radioactivity. Anterior static images with 200,000 counts were acquired and were stored in a 256 $\times$ 256 matrix. For ^{131}I-Tx imaging, a medium-energy parallel-hole collimator was used, and the energy spectrum was adjusted to 364 kiloelectron volt (keV), with a 20% window. For the ^{99m}Tc-labeled RBC imaging, a low-energy all-purpose collimator was used, and energy spectrum was adjusted to 140 keV, with a 20% window. Visual and quantitative analyses were performed on anterior static images.

All scans were evaluated by the same physician without knowledge of the implant site. Findings were considered positive if focal uptake of radioactivity at the implant site exceeded uptake at the opposite site. For quantitative analysis, regions of interest were drawn manually on lesion and contralateral side. Lesion-to-background ratio was calculated by total count of regions of interest. The implants then were surgically removed and fixed in 10% formalin for histological examination (Fig. 1). When the images, which were acquired after the injection, showed no accumulation of radioactivity in the implant sites at any time point, that is, were negative, radioactivity of the implants was measured externally by using a sensitive gamma probe (Europrobe, Eurorad Inc., Strasbourg, France), and the results were given as counts per minute (cpm).

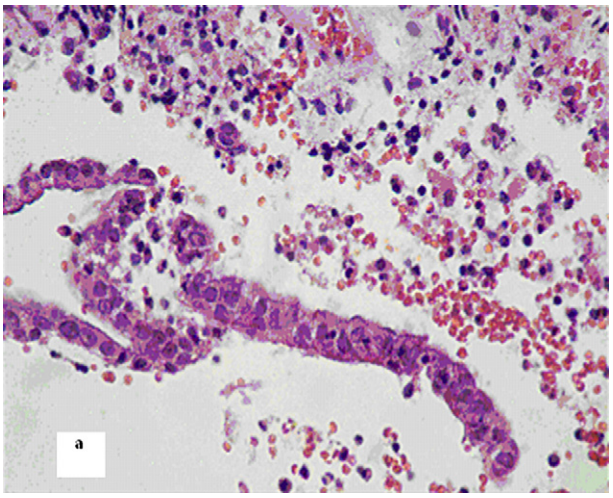
Statistical analyses were performed by using SPSS (version 13.0; SPSS Inc., Chicago, IL). Lesion–background ratios of focal hyperactive areas were compared by using the Mann-Whitney *U* test. A *P* value of $< .05$ was considered to be statistically significant. The study was approved by the institutional review board.

RESULTS

No adverse reactions or side effects were observed after administration of ^{99m}Tc-RBC. The scintigraphic images of ^{99m}Tc-RBC–injected rats were diagnostically adequate (Fig. 2). Images acquired after injection of ^{99m}Tc-RBC showed the implant in as soon as 4 hours. Focal hyperactive areas were scintigraphically documented in 11 (73.3%) of 15 rats. These rats also had macroscopic and histopathologic evidence of endometriosis.

FIGURE 1

Implanted tissue showing endometrial fragments and acute hemorrhage (hematoxylin-eosin stain; original magnification, $\times$200).



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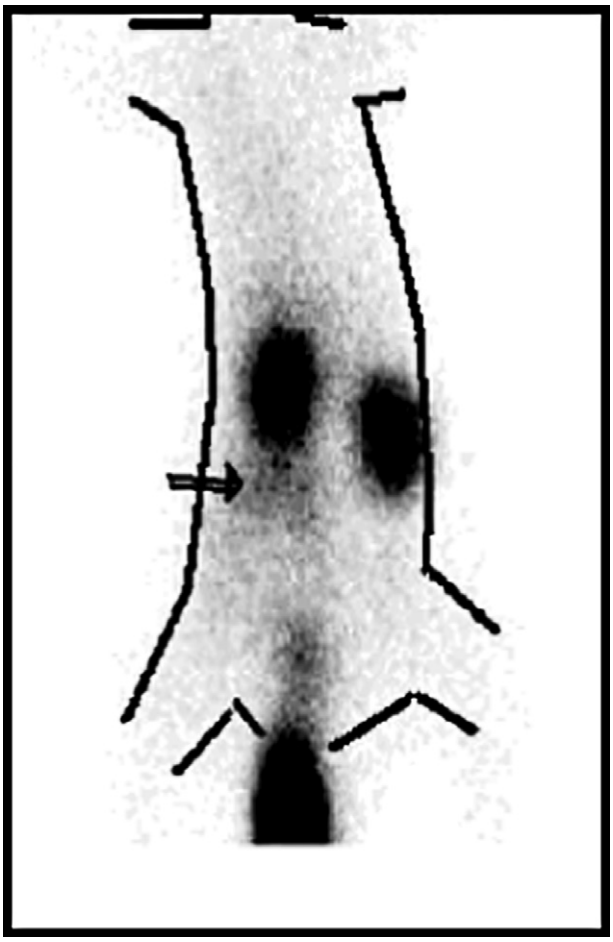
Successful implants showed disintegrating endometrial tissue (Fig. 1) with a nearby macrophage collection that contained hemosiderin pigment. Two of the 11 images were of low density, and these rats had small lesions macroscopically. Therefore, scintigraphic imaging characteristics of ^{99m}Tc-RBC strongly depended on the implant size and on bleeding within the implants. When the endometrial implants were classified into four groups according to their size (12), there was a significant positive, but subjective, correlation between the size of the implants and the subjective image quality with ^{99m}Tc-RBC.

Four rats with negative scintigraphy (26.7%) had nonspecific uptake in the implant site. These rats also underwent laparotomy, and macroscopic examination revealed inadequate vascularization and small implants, which were covered with adhesions. Histopathologic examination of two of four implants revealed evidence of infection. The lesions in the remaining two rats were negative for infection but were positive for histological evidence of endometriosis. The infected implants were small (grade 2) and were characterized by large accumulations of polymorphonuclear leukocytes around abundant areas of necrosis.

The activity, in other words, concentration, of ^{99m}Tc-RBC in the implant at 4 hours after injection was high, and visualization of endometriotic lesions was better with ^{99m}Tc-RBC than with ^{131}I-Tx. The images in group 2, which were acquired after the ^{131}I-Tx injection, showed no accumulation of radioactivity in the implant sites at any time point. The uptake of ^{131}I-Tx was much less than

FIGURE 2

Distribution of activity in the rat 4 hours after injection of ^{99m}Tc-RBC. *Arrow* shows focal hyperactive area in implant site.



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that of ^{99m}Tc-RBC. On further measurement that was performed with a gamma probe, implanted sites had a radioactivity level (mean ± SD) of 190.8 ± 32.6 cpm, and control sites had a radioactivity level of 185.3 ± 30.5 cpm. Radioactivity level in the excretory routes (bladder and kidney) for ^{131}I-Tx was 263.3 ± 24.2 cpm and 260.0 ± 47.3 cpm, respectively. Lesion-to-background ratios of focal hyperactive areas on ^{99m}Tc-RBC scintigraphy (2.015 ± 0.619) was higher than on ^{131}I-Tx scintigraphy (1.07 ± 0.16; $P<.05$).

DISCUSSION

Radioimmunodetection, a technique that was initially developed to identify malignant tissue, has diverse applications such as myocardial imaging, thrombi, inflammation, and atherosclerotic plaques (8). In radioimmunodetection, a specific antibody against the investigated cell type is labeled with a radionuclide agent and is called the tracer. The tissue or organs of interest then are scanned with a gamma camera to detect any excess accumulation of the tracer. In this context, a tracer for the endometriotic tissue may be proved to be useful in the diagnosis and assessment of endometriosis. This hypothesis was investigated in two case reports (9, 10). In these studies ^{131}I- or ^{111}In-labeled OC-125 F(ab′)2 anti-CA-125 was used to detect pelvic and pulmonary sites.

In the present study, we studied the performance of ^{99m}Tc-RBC and ^{131}I-Tx in a well-established animal model of peritoneal endometriosis. We did not prefer a CA-125–based tracer because its existence has not been studied in experimentally induced disease. Instead of it, we used ^{99m}Tc-RBC, an agent that frequently is used in minute quantities of bleeding. This agent has been used to detect bleeding that is ongoing at a rate as low as 0.1 mL/min, especially in gastrointestinal hemorrhages (14, 15). A hemorrhage, per se, is a key element in endometriosis that justifies the use of ^{99m}Tc-RBC.

The ^{99m}Tc-RBC scintigraphy was applied to visualize hemorrhage in endometrial implants. We successfully imaged the majority of the implants with ^{99m}Tc-RBC scintigraphy. Because no direct method has been developed to evaluate ongoing bleeding in the implant, we studied the relation between the size of the lesions and ^{99m}Tc-RBC images with the assumption that ongoing bleeding would be greater in larger implants (Fig. 1). It was not surprising, then, to find that the uptake of ^{99m}Tc-RBC was indeed greater in the larger implants. The existence of the hemosiderin-laden macrophages near the endometrial tissue in histological examination of the implants from RBC-injected rats showed the presence of bleeding, as radioimmunodetection had implied.

This study demonstrates that although the radionuclide image can accurately assess most of the endometriotic lesions, it does not accurately estimate the presence of some implants. Scintigraphy with ^{99m}Tc-RBC failed to show macroscopically and histologically evident implant sites in four animals. This unfavorable feature also could be explained by the restricted angiogenesis, which would have resulted in lack of significant implant accumulation of the tracer, as well as by the absence of microbleeding. The sensitivity of the technetium scan is reportedly 20%–95% (14, 15). The bleeding site can be identified accurately when intraluminal accumulation of ^{99m}Tc-RBC is observed during the dynamic phase of scanning. Although nuclear scintigraphy is sensitive enough to diagnose ongoing bleeding at a rate as low as 0.1 mL/min, it is not highly accurate in locating the bleeding point (14, 15). Aberrant or ineffective vascular anatomy can disturb the circulation of small endometrial implants. Another possibility is that bleeding may present in an uncommon clinical fashion in grade 1 and 2 implants. Grade 3 and 4 implants were always associated with a positive radionuclide image, and a positive image

was considered to represent sufficient neovascularization and implant bleeding.

Scintigraphy with ^{99m}Tc-RBC allowed a visual differentiation between grade 1 and 4 vesicular lesions within 4 hours after injection. In addition, image quality improved further from grade 1 to 4 endometriotic lesions. The higher uptake of ^{99m}Tc-RBC in the grade 3 and 4 vesicular lesions was mainly the result of an increased uptake of ^{99m}Tc-RBC in the hypervascularized implants. This led to a more accurate visual differentiation between small and large vesicular lesions. Furthermore, the excellent performance of ^{99m}Tc-RBC was illustrated by its low uptake in the implant-free peritoneal wall. Apparently, bleeding within implant is an important reason for the dramatically better performance of ^{99m}Tc-RBC compared with ^{131}I-Tx in scintigraphic imaging of peritoneal endometriosis.

Accumulation of the ^{99m}Tc-RBC in endometriotic lesions can vary with respect to degree of ongoing bleeding, lesion size, and angiogenesis. Angiogenesis, the formation of new blood vessels, plays an important role in the formation and growth of endometriotic lesions (16). Although some endometriotic lesions show prominent vascularization and often are red in appearance because of hemorrhagic admixture, some implants show weak vascularization and are colored white or yellow (17). Nisolle et al. (16) showed that red endometriotic lesions were better vascularized and that this was the result of a larger vessel diameter rather than of the number of vessels, which was significantly higher in black lesions. Such sites of inadequate angiogenesis would not be expected to show increased uptake on the radionuclide image. The sites of angiogenesis formation should show increased uptake. Thus, a positive image of ^{99m}Tc-RBC scintigraphy indicates sufficient angiogenesis, and a negative image means that effective blood supply has not been acquired.

When evaluating the microbleeding pattern of endometriosis tissue, it is important to determine whether a hemorrhage is present, at all types and all stages of the disease. It is known that ectopic endometrium may not necessarily be synchronous with normal endometrium. In one study, 43% of endometriosis tissue was out of phase (18). In another report, only 13% of endometrial implants were histologically synchronous with the corresponding intrauterine endometrium (19). Hence, negative scintigraphy may be the result of the intermittent nature of implant bleeding. These results suggest that the bleeding of endometriotic implants is unpredictable and inconsistent.

Adhesions at the site of implants may cause decreased angiogenesis and could be the main factors leading to nonspecific decreased uptake in these implants. In endometrial implants with no infection or adhesion, ^{99m}Tc-RBC remains in the cells, making it easier to detect by scintigraphy. In this study, ^{99m}Tc-RBC scintigraphy tended to be positive in cases in which infection was negative, whereas scintigraphic images were negative in cases in which infection or adhesion was positive. Therefore, we hypothesized that in vivo interaction with polymorphonuclear leukocyte (PNL) at the site of infection, combined with inadequate vascularization in the implant, can lead to reduced uptake and prevent visualization of the endometriotic implants. Differences in histopathology between normal implants and implants with infection may (at least in part) explain the difference in scintigraphic performance.

Scintigraphic study with ^{131}I-Tx did not yield satisfactory images in any of the subjects. Although Kadaba and Simpson (20) have reported that tamoxifen is effective in regressing endometrial implants in rats with experimentally induced endometriosis, many other investigators, including Bergqvist et al. (21), have shown that endometriotic tissue contains lower concentrations of estrogen and P receptors than does normal endometrium. Therefore, that negative outcome can be explained on the basis of the weak estrogen receptor content of implants.

What clinical usefulness does radionuclide imaging have in peritoneal endometriosis? Studies elsewhere have suggested that it may be useful as an adjunct to other imaging procedures in evaluating pulmonary endometriosis (9, 10). It may also be useful in demonstrating deep endometrial implants, particularly in areas that are not seen well on laparoscopy. The radionuclide image may detect areas of early implant involvement that are not yet evident on laparoscopy and that, when combined with laparoscopy, may help to assess the extent of disease. The radionuclide image may be useful in patients who have grade 3 and 4 vesicles; demonstration of peritoneal implants would presumably indicate that the patient is at a higher risk for endometriosis and may warrant a more invasive diagnostic approach, such as laparoscopy.

We introduced ^{99m}Tc-RBC as such a new agent for scintigraphic diagnosis of peritoneal endometriosis. Although the number of implants studied was limited, the findings in our study using ^{99m}Tc-RBC scintigraphy may contribute to development of a unique approach to the noninvasive diagnosis of peritoneal endometriosis. In the future, implant-specific drugs such as aromatase inhibitors may be developed to demonstrate lesions and can be used in conjunction with ^{99m}Tc-RBC scintigraphy as an imaging method to visualize endometrial implants. Despite these several limitations, we consider that ^{99m}Tc-RBC scintigraphy may be a cost-effective and useful procedure to guide the surgeon during laparoscopy. Our results also suggest that negative scintigraphic images should not be interpreted as absence of endometriotic disease, thus reducing the sensitivity of test.

The correct approach for the management of endometriosis is still unclear (22). Novel techniques that are investigated for easy and accurate diagnosis of endometriosis, for instance, biochemical markers, may show only the

presence or absence of endometriosis. The present study adds a new scintigraphic technique, in vivo ^{99m}Tc-RBC, to the relevant diagnostic armamentarium. The tracer used has an ease of preparation, early good image quality, and low radiation burden. It is superior to biochemical markers because it also is able to detect the location and extent of implants. It may also give functional information by the measurement of activity vs. time. The noninvasive nature of the scintigraphy is its major advantage over endoscopic techniques, making it possible to perform repeat imaging to monitor the progress of the disease and the effect of treatment.

Although the ^{99m}Tc-RBC image skips a few implant sites, it can be useful for the detection and monitoring of endometriotic implants. We have been able not only to determine the presence of an endometriosis but also to evaluate the size, location, and function of implants. We documented the superior performance of ^{99m}Tc-RBC compared with ^{131}I-Tx for the evaluation of endometriosis. The primary method of evaluating peritoneal involvement by endometriosis is the patient's history and laparoscopy. Further applications of radionuclide imaging in peritoneal endometriosis may follow if the pathophysiology can be explained that results in positive images at some endometrial implants and in negative images at others.

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