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Rat Endometrial Stromal Cells Express the Gap Junction Genes Connexins 26 and 43 and Form Functional Gap Junctions during In Vitro Decidualization[1]

C.E. Orlando-Mathur [2,3] J.F. Bechberger [4] G.S. Goldberg [4] C.C.G. Naus [4] G.M. Kidder, [5] and T.G. Kennedy [3,6]

Departments of Physiology,[3] Anatomy, [4] Zoology, [5] and Obstetrics and Gynaecology [6]
The University of Western Ontario, London, Ontario, Canada N5A 5A5

ABSTRACT

Gap junctions form between rat endometrial stromal cells as they undergo decidualization. We have examined the steady-state levels of the gap junction transcripts, connexins 26 and 43 (cx26 and cx43), during artificially induced decidualization in vivo and found that they have a temporal pattern similar to that observed in pregnancy. An in vitro model of decidualizattion was then used. Endometrial stromal cells from rat uteri sensitized for decidualization were cultured for 24, 48, or 72 h before total RNA was extracted and subjected to Northern blot analyses to determine the steady-state levels of cx26 and cx43 transcripts. The analyses revealed that cx26 transcript steady-state levels decreased, whereas those for cx43 increased, from 24 to 72 h. Using an anti-cx43 antibody, punctate immunofluorescent signals were observed around the periphery of the cells, suggesting that cx43 had been assembled into membrane plaques. The presence of functional gap junctions between the cells was determined in vitro by two dye-coupling methods: preloading and scrape-loading. Calcein (995 Da) and a membrane-bound dye, dioctadecyl-3,3,3',3-tetramethylindocarbocyanine perchlorate (933 Da), were preloaded into 5% of the endometrial stromal cells before plating. The percentage of preloaded cells that transferred calcein to adjacent cells increased from 10% at 3 h after plating to 40% at 6 h. To determine whether or not cells maintain the ability to dye-couple throughout the culture period, carboxyfluorescein (CF; 376 Da) and rhodamine dextran (9.3 kDa) were introduced into cells by scraping the cells with a scalpel, and the distribution of dyes was determined 20 min later. In cells cultured for 24, 48, or 72 h, only CF was transferred to cells distal to the scrape line. The results from these experiments suggest that stromal cells can dye-couple throughout the culture period (3-72 h) and indicate that functional gap junctions form between endometrial stromal cells as they undergo decidualization in vitro.

INTRODUCTION

In species in which implantation of the embryo is invasive, such as humans and rodents, implantation of the embryo in the receptive endometrium triggers a series of responses collectively called decidualization. During decidualization the endometrial stromal cells undergo proliferation and differentiation into decidual cells. As the cells decidualize they increase in number and volume, thereby reducing the intercellular space. Coincidental with the decrease in intercellular space is the formation of cell contacts such as gap junctions [1].

In the decidua, "specialized junctions," now known to be gap junctions [2], were first identified in the mouse 13]. Subsequently, decidual gap junctions were reported in the hamster [4] and the rat [5-8], and in human predecidual cells in vivo [9] and in vitro [10, 11]. All rodent species studied exhibit the following temporal and spatial pattern of gap junction appearance during decidualization, whether in pregnancy or in response to an artificial deciduogenic stimulus [7]. Prior to decidualization, no gap junctions are found between endometrial stromal cells. The presence of gap junctions between endometrial stromal cells 24 h after the initiation of implantation of the embryo is one of the earliest signs of decidual cell transformation. Thereafter there is a progressive increase in the number of gap junctions between decidual cells until the antimesometrial cells regress to form the decidua capsularis.

Gap junction channels consist of hexameric aggregates of individual proteins called connexins [12]. Connexins are identified according to the molecular mass of the protein as predicted from the cDNA; for example, connexin26 (cx26) has a molecular mass of 26 kDa. In pregnant rats, steady-state levels of transcripts for cx26 and cx43 were determined during decidualization by means of Northern blot analysis 113, 14] and found to be relatively abundant, with expression coinciding with the onset of decidualization. During artificially induced decidualization, steady-state levels of cx26 and cx43 transcripts were greater in the stimulated uterine horn undergoing decidualization than in the nonstimulated horn [15]. Thus, for the endometrium, cx26 and cx43 are decidualization-associated transcripts.

The biological role of decidual gap junctions is unknown. During implantation in the rabbit, luminal epithelial cells become junctionally coupled, possibly to coordinate the response of the epithelium [16]. A similar function may occur in the antimesometrial decidua to synchronize the growth and subsequent regression of the decidua 13, 5, 8].

The purpose of the present study was to determine whether or not decidual gap junctions are functional. Both in vivo and in vitro models of decidualization were used. Since previous investigators found that the steady-state lev-

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[2]Correspondence: C.E. Orlando-Mathur, Department of Obstetrics and Gynaecology, University Hospital, 339 Windermere Rd., London, ON, Canada N6A 5A5. FAX: (519) 663-3388.

els of cx26 and cx43 transcripts increase during decidualization [13-15], we focused our investigation on these two connexins. The temporal pattern of steady-state transcript levels for cx26 and cx43 were examined by Northern blot analyses during artificially induced decidualization in vivo and were compared to those found during in vitro decidualization. Dye-coupling methods were employed to determine whether decidual gap junctions are functional.

MATERIALS AND METHODS

Animals

Female Harlan Sprague-Dawley rats (200-250 g; Indianapolis, IN) were housed under light (lights-off from 1900--0500 h)- and temperature-controlled conditions with free access to food and water.

Treatment of Animals

Rats weighing 200-225 g were ovariectornized under ether anesthesia and allowed at least 4 days to recover from surgery. To obtain rats sensitized for the decidual cell reaction, estradiol-17β and progesterone were dissolved in sesame oil and administered as indicated in Figure 1 [17].

Decidualization In Vivo

Between 1000 and 1200 h on the equivalent of Day 5 of pseudopregnancy, under ether anesthesia, rats were given a bilateral deciduogenic stimulus of 100 μl of sesame oil injected into each uterine horn caudal to the uterotubal junction. A ligature was tied around the end of the uterus caudal to the site of injection to prevent leakage of oil through the injection site. Rats were killed at various times thereafter. Some animals did not receive the deciduogenic stimulus ("nonstimulated") and served as a control to differentiate the effects of decidual induction and duration of exposure to steroid hormones.

Decidualization In Vitro

Rats were killed by decapitation on the morning of the equivalent of Day 5 of pseudopregnancy. Uteri were excised. above the cervices and trimmed of adherent tissue. The uterine horns were split longitudinally and placed in Hanks' balanced salt solution without Ca^{2+} and Mg^{2+} (HBSS; Gibco-BRL, Burlington, ON, Canada). Endometrial stromal cells were isolated by a modification of the technique of McCormack and Glasser [18]. In summary, the horns were first treated with dispase II (6 mg/ml; Boehringer-Mannheim, Dorval, PQ. Canada) and pancreatin (2.5%; Gibco-BRL) to isolate epithelial cells and then with collagenase (type II, 0.5 mg/ml; Sigma Chemical Company, St. Louis, MO) to isolate stromal cells. The stromal cells were suspended in Dulbecco's Modified Eagle's medium (DMEM)-F12 (Gibco-BRL) containing 10% heat-inactivated charcoal-stripped fetal calf serum (FCS; Gibco-BRL) and counted; then 24-well plates (Becton-Dickinson, Lincoln Park, NJ) were seeded with 5 X 10^5 cells per well in 0.5 ml of DMEM-F12 and 10% FCS. After a 2-h stromal attachment period, the medium was removed and replaced with fresh medium [19].

Epithelial Cell Separation

To aid in the interpretation of results it was necessary to determine the cell types that express cx26 on the equivalent of Day 5 of pseudopregnancy. Endometrial stromal cells and epithelial cells were separated either enzymatically (see above) or mechanically. For the mechanical separation of cell types, the method of Bitton-Casimiri et al. [20] was employed. The separated cells were placed in guanidinium isothiocyanate (GTC) solution (4 M guanidine thiocyanate, 0.5% sarkosyl N-laurosarcosine, 25 mM sodium citrate [pH 7.0], 0.1% antifoam A, and 5% β-mercaptoethanol) and homogenized with a Teflon-glass homogenizer; the RNA was then extracted (see next section).

Northern Blot Analyses

For in vivo tissue preparations, endometrium was separated from myometrium by gentle scraping [21]. The endometrium was placed in GTC solution and homogenized with a Teflon-glass homogenizer. To extract RNA from cultured cells, the medium was removed, GTC solution was placed in the wells, and the cells were detached by rubbing the bottom of the wells with the end of the sterile plunger from a 1-ml tuberculin syringe. RNA was subsequently isolated by extraction with phenol/chloroform [22].

Standard procedures for Northern blot analyses were conducted as outlined by Sambrook et al. [23] with some modifications. Total RNA (10-20 μg) was denatured in 6% formaldehyde/50% formamide in MOPS buffer (20 mM 3-[N-morpholinolpropanesulfonic acid [MOPS], 5 mM sodium acetate, 1 mM EDTA, pH 7.2) at 65°C for 15 min. The RNA was electrophoresed in a formaldehyde denaturing gel with RNA size standards and stained with ethidium bromide to determine RNA integrity. RNA was transferred to Hybond (Amersham, Oakville, ON, Canada) nylon membrane by capillary transfer for 24 h in 20-strength SSC (3 M NaCl, 0.3 M sodium citrate). After transfer the RNA was cross-linked to the membranes by exposure to UV light.

Probes were labeled by random hexamer labeling (Random Primers DNA Labeling System, Gibco-BRL) in the presence of [α-^{32}P]dCTP (Amersham). The cx26, cx43, and 18S cDNA probes were gifts from BJ. Nicholson (State University of New York, Buffalo, NY), E. C. Beyer (Washington University, St. Louis, MO), and G.L. Hammond (University of Western Ontario, London, ON, Canada), respectively, and used according to their reported methods ([24-26], respec

tively). The membranes were prehybridized at 65°C for 2 h in Northern hybridization solution (0.9 mM sodium pyrophosphate, 1 M NaCl, 1% SDS solution, 10-strength Denhardt's reagent and 80 μg/ml salmon sperm DNA) and then hybridized with the labeled probe for 18-24 h in the same solution. The membranes were washed at 65°C twice in double-strength SSC/0.1% SDS for 30 min and then twice in 0.2-strength SSC/0.1% SDS for 30 min. Exposure of x-ray film to the blots was conducted at - 70°C with intensifying screens. Hybridization signals were evaluated by image analysis (Mocha Image Analysis; Jandel, Corte Madera, CA) and normalized relative to 18S rRNA to account for unequal loading and transfer of RNA. To remove probes from the membranes, the membranes were immersed for 2 h in 500 μl of 1 mM Tris (pH 8), 1 mM EDTA, and 0.1-strength Denhardt's reagent at 75°C. Each membrane was probed a maximum of three times.

Steady-state levels of cx26 and cx43 were examined on the equivalent of Days 5, 6, 8, and 10 of pseudopregnancy in stimulated and nonstimulated uterine horns. Three blots from three independent experiments from cells cultured for 24, 48, and 72 h were probed with the cx43 cDNA probe. These blots were reprobed with cx26 along with a fourth preparation of RNA from cultured cells.

Preparation of Glass Coverslips for Fluorescence Studies

Cells were cultured on glass coverslips in 24-well plates (12 mm^2, 0.13-0.17 mm thick; Fisher Scientific, Pittsburgh, PA) in the experiments that utilized fluorescent compounds, since plastics autofluoresce. Coverslips were degreased with acetone and ethanol, etched with nitric acid for 6 h, and then autoclaved. To determine the relative plating efficiencies of cells on glass and plastic, the cells were liberated by trypsin digestion (0.5%) and then counted 2 h after plating. No differences were observed between the plating efficiencies.

Immunocytochemistry

To determine whether cx43 is assembled into membrane plaques of cultured endometrial stromal cells, indirect immunofluorescence was utilized with CT-360 antibody, generously supplied by Dale Laird (McGill University, Montreal, PQ Canada). This antibody was raised against a synthetic peptide corresponding to the C terminus (360-382) of cx43 [27, 28]. Cells grown on glass coverslips for 40 h were fixed in cold methanol (- 20°C) for 10 min and then washed five times with PBS, with the last wash being left on for 15 min. The cells were incubated at room temperature (RT) in a blocking buffer containing 1% BSA and 10% goat serum in PBS for 1 h; this was followed by a 1-h incubation with the primary antibody or preimmune serum (1:250 dilution in 1% BSA in PBS). The cells were then washed five times with PBS, with the last wash being left on overnight at 4°C. The next day the cells were incubated for 1 h at RT with fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit antibody (ICN Biomedicals, Montreal, PQ, Canada; 1:200 dilution in 1% BSA in PBS) and then washed five times with PBS with the last wash being left on for 5 h at 4°C to reduce nonspecific staining. The coverslips were mounted onto slides with FITC-Guard (Testog Inc., Chicago, IL) and then viewed via a Zeiss epifluorescence microscope equipped with filter set 17 (exciter BP485 nm, barrier 515-565 nm; Carl Zeiss, Thornwood, NY).

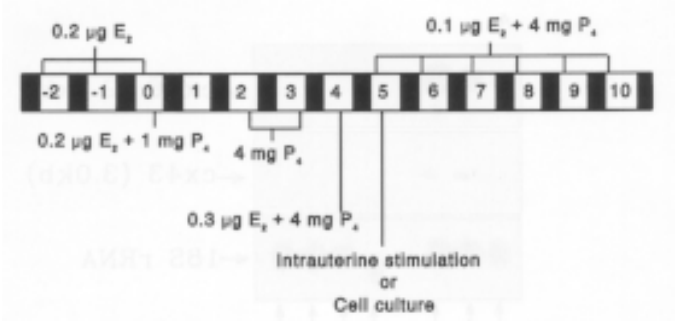

FIG. 1. Schematic representation of the treatment protocol to obtain rats sensitized for the decidual cell reaction. Steroids were dissolved in sesame oil and administered s.c. For decidualization in vivo, some of the animals were given bilateral intrauterine injections of 100 μl of sesame oil around noon on the equivalent of Day 5 of pseudopregnancy (S). The animals that did not receive the deciduogenic stimulus served as "nonstimulated" controls (N). The animals were killed on subsequent days and RNA was isolated from endometrial or decidual scrapings. For in vitro decidualization experiments, the animals were killed on the morning of the equivalent of Day 5 of pseudopregnancy, and the cells were isolated and cultured. The open areas represent lights-on; dark areas, lights-off.

Preloading

The technique of preloading [29, 30] was used to determine the ability of the endometrial stromal cells to dye-couple 3 and 6 h after plating. Before the final suspension of cells was plated, 5% of the cells were preloaded with two dyes: dioctadecyl-3,3,3',3-tetramethylindocarbocyanine perchlorate (DiI, 933 Da; Molecular Probes, Eugene, OR) and calcein-acetoxymethyl ester (Calcein-AM, 994 Da; Molecular Probes). To load the dyes, cells were suspended and incubated at 2.5 X 10^6 cells per milliliter in sterile isotonic glucose containing 0.5% DiI and 2.0% Calcein-AM for 1 h at 37°C. To remove the free dyes from the cell suspension after the incubation, the cells were centrifuged and resuspended twice in DMEM-F12 and 10% FCS. Cell viability of the preloaded cells was estimated to be approximately 50%. Preloaded cells (25 000 viable cells per well) were plated with nonloaded cells (475 000 per well). After the differential attachment period of 2 h, the medium was removed and replaced with fresh medium. The cells were examined for dye distribution at 3 and 6 h postplating by means of a Zeiss epifluorescence microscope; filter set 15 (exciter BP546 nm, barrier LP590 nm) was used for DiI and filter set 17 for calcein. The numbers of preloaded cells (identified on the ba-

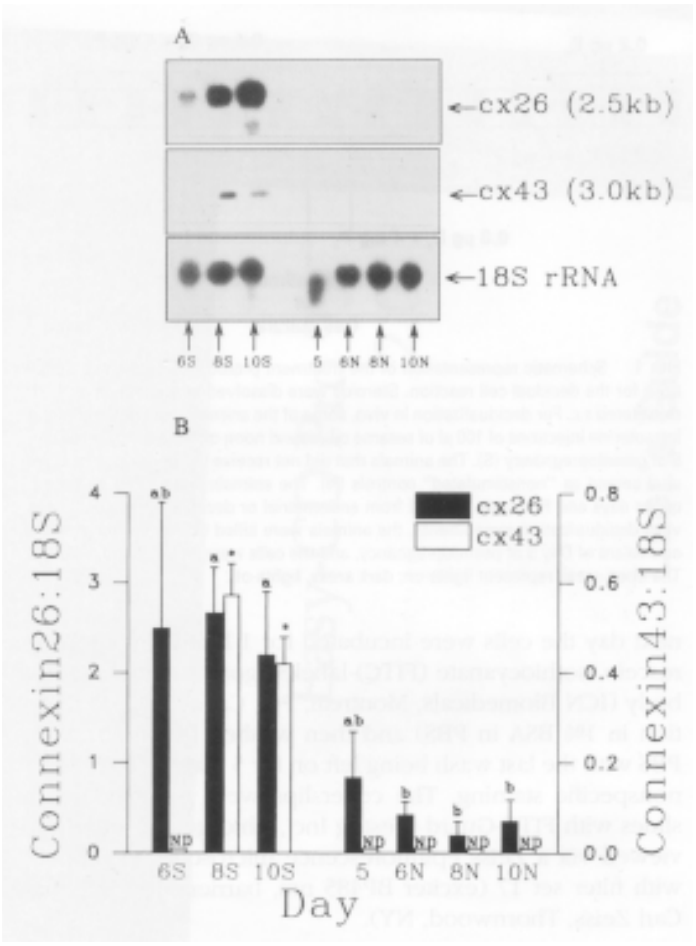

FIG. 2. Northern blot analyses of cx26 and cx43 steady-state levels during artificially induced decidualization. **A)** Representative photographs of Northern blot analyses for 18S rRNA and cx26 and cx43 transcripts. **B)** Image analyses for cx26 and cx43 during decidualization in vivo. The hybridization signals were normalized relative to 18S rRNA to account for unequal loading and transfer of the RNA. RNA (~20 μg/lane, n = 3) was isolated on Days 5, 6, 8, and 10 from stimulated (S) and nonstimulated (N) endometrial/decidual cells. Exposure times were cx26, 10 days; cx43, 7 days; and 18S, 8 h. Data are expressed as mean ± SEM. For cx26, bars containing different letters are significantly different from each other ($p < 0.05$). Cx43 was not detected (ND) in Day 5 and 6S cells and in all N endometrial cells.

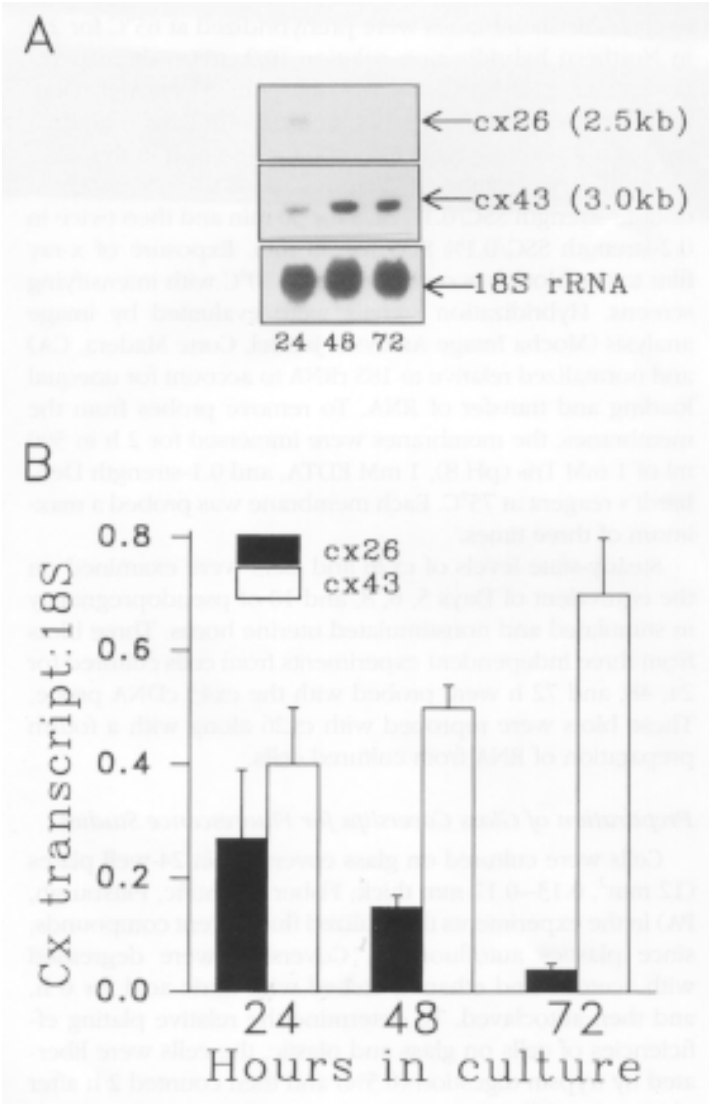

FIG. 3. Northern blot analyses of cells undergoing in vitro decidualization. **A)** Representative photographs of 18S rRNA and cx26 and cx43 transcripts. RNA (~10-15 μg/lane) was isolated from endometrial stromal cells after 24, 48, or 72 h in culture. Exposure times for the transcripts were cx26, 10 days; cx43, 6 h; and 18S rRNA, 8 h. Three blots from three independent experiments were analyzed for cx43, and an additional blot was probed with cx26. **B)** Image analyses were conducted, and the hybridization signals were normalized relative to 18S rRNA to account for unequal loading and transfer of the RNA. Data are expressed as mean + SEM.

sis of DiI fluorescence) coupled to non-preloaded cells (no DiI fluorescence, calcein fluorescence present) were determined. The degree of coupling was estimated by determining the numbers of calcein-positive/DiI negative cells adjacent to DiI-positive cells. Four independent experiments were conducted, and a minimum of 200 DiI-loaded cells at each time point in an experiment were assessed.

Scrape-Loading

The method of scrape-loading was used to determine whether the cells exhibited dye coupling after 24, 48, and 72 h in culture [31]. Medium was removed from the wells and replaced with 500 μl of 0.1% 5- and 6-carboxyfluorescein mixed isomers (CF, 376 Da; Molecular Probes) and 0.1% rhodamine B isothiocyanate-dextran 10S (RD, 9.3 kDa; Sigma) in HBSS. A scalpel blade was scraped down the middle of the well to load the dyes into the wounded cells. The dye-containing solution was removed 2 min later, and the cells were rinsed to remove detached cells and to reduce the background fluorescence. At various times after scrape-loading, the cells were examined for dye distribution with a Zeiss epifluorescence microscope; filter set 17 was used for CF and set 15 for RD.

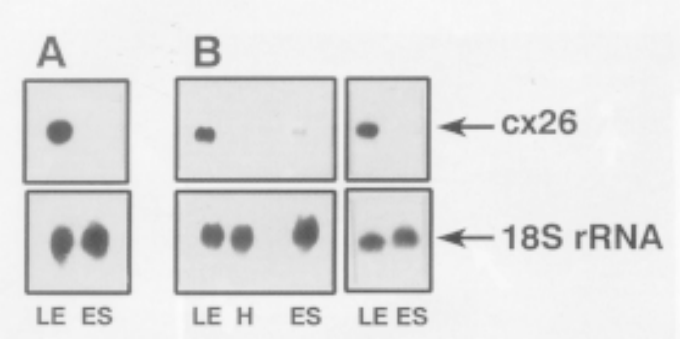

FIG. 4. U26 and 18S rRNA transcript steady-state levels in the luminal epithelium (LE) and endometrial stromal (ES) cells separated mechanically **(A)** or enzymatically **(B)**. Heart (H) RNA was analyzed as a negative control.

Statistical Analysis

The data are presented as mean ± SEM of 3 or 4 independent experiments. The image analysis data from the Northern blots were log transformed prior to statistical analysis to reduce heterogeneity of variance [32]. The significance of the effects of time on connexin mRNA steady state levels was determined by analyses of variance. When significant interactions were found, in factorial analysis of variance, Duncan's New Multiple range test was used for group comparisons [33]. The preloading experiment was analyzed by RxC contingency tests followed by individual chi-square tests [32].

RESULTS

Cx Transcript Steady-State Levels during Artificially Induced Decidualization In Vivo

The temporal patterns of cx26 and cx43 transcript steadystate levels were examined on the equivalent of Days 5, 6, 8, and 10 of pseudopregnancy in stimulated and nonstimulated uterine horns (Fig. 2). As indicated by image analyses (Fig. 2B), cx26 transcript steady-state levels were low in the endometrium on the equivalent of Day 5 of pseudopregnancy and increased thereafter by more than 2-fold in the stimulated horns as decidualization progressed. By contrast, in the nonstimulated tissue the signal intensity remained low. Cx43 transcripts were detected in the stimulated uterine horns only on Days 8 and 10.

Cx Transcript Steady-State Levels during Decidualization In Vitro

During decidualization in vitro, both cx26 and cx43 were expressed (Fig. 3). In contrast to findings for in vivo decidualization, cx26 transcript steady-state levels decreased linearly with time ($p < 0.01$, analysis of variance using orthogonal polynomials). Cx26 steady-state levels were significantly less ($p < 0.02$) at 72 h compared to 24 and 48 h.

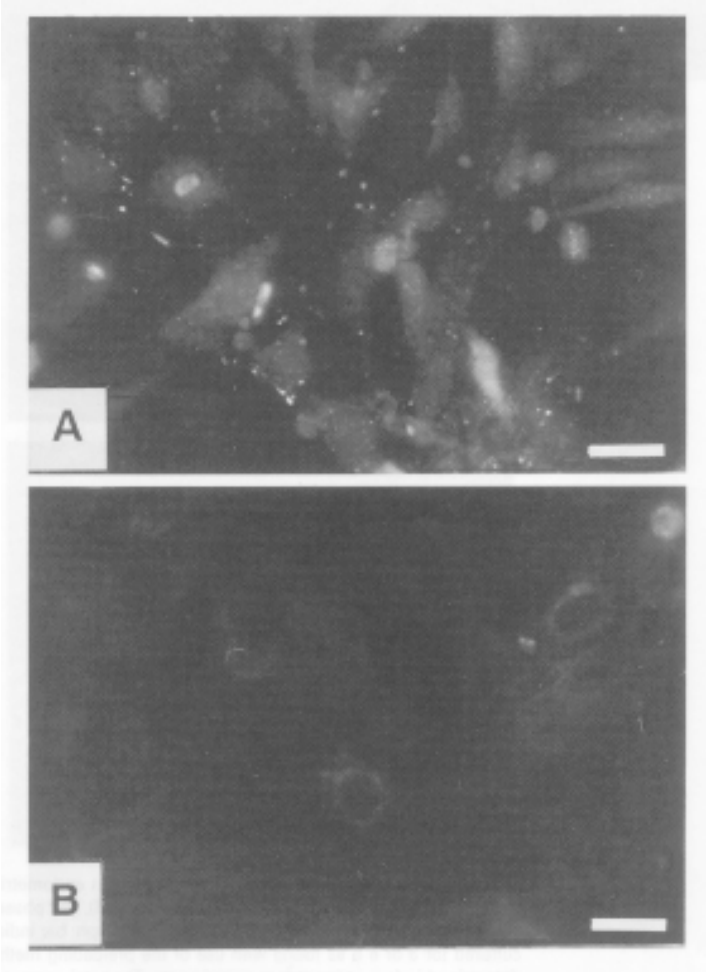

FIG.5. Immunolocalization of cx43 during invitro decidualization at40 h in culture. The results with anti-cx43 antibody (A) and preimmune serum (B) are shown. Bar indicates -10 gm.

Analysis of variance of the cx43 image analysis data using orthogonal polynomials revealed a trend ($p = 0.052$) for the cx43 steady-state levels to increase in a linear fashion with time in culture.

Cx26 Northern Blot Analysis: Epithelial Vs. Stromal Cells

As determined by Northern blot analysis, luminal epithelial cells, whether separated enzymatically or mechanically, had higher steady-state levels of cx26 transcripts than endometrial stromal cells (Fig. 4).

Cx43 Immunofluorescence

Endometrial stromal cells cultured for 40 h were examined for cx43 immunofluorescence (Fig. 5). Punctate fluorescent signals were observed around the periphery of the cells with use of the antibody (Fig. 5A) but not its preimmune serum (Fig. 513). Diffuse fluorescent signals were ob-

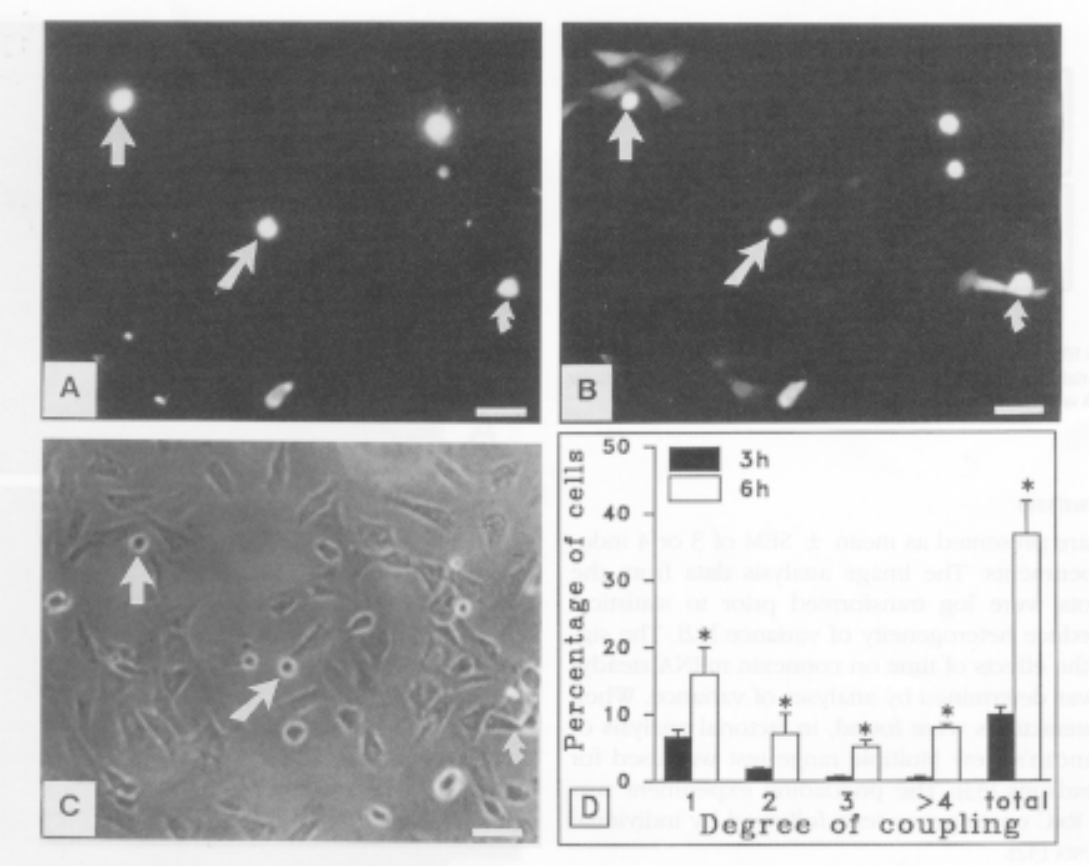

FIG. 6. Representative example of dye coupling in endometrial stromal cells cultured for 6 h as detected via the preloading method. DiI fluorescence **(A),** calcein fluorescence **(B),** and phase-contrast (C) photomicrographs of the same field are shown. The arrows indicate the same cell in each photomicrograph; bar indicates ~20 μm. D) Representation of the degree of coupling of cells cultured for 3 or 6 h as found with use of the preloading method. The percentage of cells coupled to 1, 2, 3, or > 4 cells was determined in four independent experiments. The total percentage of cells coupled to at least one cell is also represented. Each point represents the mean ± SEM.

served inside the cells. These signals were slightly stronger than those obtained by means of the preimmune serum.

Preloading

A representative example of the dye distribution in cells at 6 h with use of the preloading method is shown in Figure 6. Photomicrographs showing DiI fluorescence (Fig. 6A), calcein fluorescence (Fig. 6B), and the cells under phase contrast (Fig. 6C) are shown. Calcein fluorescence and DiI fluorescence were found in the same cells. Often, only calcein was found also in cells adjacent to the DiI/calcein-loaded cells. This presumed coupling was quantified by determining the percentage of DiI-loaded cells coupled to 1, 2, 3, or > 4 calcein-only-cells (Fig. 6D). RxC contingency tests revealed that the independent experiments were not significantly different ($p > 0.05$) from each other, and that at 6 h compared to 3 h the proportion of cells exhibiting coupling was significantly greater ($p < 0.01$). Coupling was observed in approximately 10% of the cells at 3 h, whereas at 6 h almost 40% of the cells exhibited dye coupling.

Scrape-Loading

A representative example of cells cultured for 24 h and then subjected to scrape-loading is depicted in Figure 7. Photomicrographs of the RD fluorescence (Fig. 7A), CF fluorescence (Fig. 7B), and cells under phase contrast (Fig. 7C) are shown. CF but not RD was found in cells contiguous with other labeled cells located distal to the scrape line. The selective transfer of CF but not RD was also observed in endometrial stromal cells cultured for 48 and 72 h (results not shown).

DISCUSSION

In this study we examined the temporal patterns of cx26 and cx43 transcript steady-state levels, cx43 immunofluorescence, and dye coupling in endometrial stromal cells undergoing decidualization; our results suggest that functional gap junctions form between these cells as they undergo decidualization.

The temporal patterns of cx26 and cx43 transcript steady

FIG.7. Representative example of endometrial cells cultured for 24 h and then scrape-loaded. RD fluorescence **(A**), CF fluorescence **(B),** and phase-contrast **(C)** photomicrographs of the same field are shown. The photomicrographs were taken 20 min after scraping.

state levels we have found during artificially induced decidualization are similar to those reported during pregnancy [13, 14]. Cx26 transcript steady-state levels increased during artificially induced decidualization (Fig. 2), as has also been reported during pregnancy. Moreover, during pregnancy it was found that cx43 transcript steady-state levels rose sharply on Day 7 and thereafter remained relatively constant. As can be seen in Figure 2, a similar pattern of cx43 steady-state levels was observed on the equivalent days of pseudopregnancy. It is already known that cx26 and cx43 are expressed during artificially induced decidualization [15], but since the patterns of cx26 and cx43 steady-state levels are similar during pregnancy and artificially induced decidualization, we conclude that embryonic factors are not required to maintain these temporal patterns.

Both cx26 and cx43 transcript steady-state levels were higher in the decidualized tissue compared to nondecidualized tissue (Fig. 2), and both cx26 and cx43 were expressed during in vitro decidualization. It is known that in the rat endometrium, cx26 and cx43 steady-state levels are regulated by estrogen and progesterone [15]. Since cx26 and cx43 transcript steady-state levels were low in the nonstimulated uterine horns and were expressed in the absence of hormones in vitro, the expression of both these transcripts may also be controlled by a factor produced during decidualization. A chemical factor that is known to affect gap junctional expression [34] and is a mediator of decidualization is prostaglandin E_2 [35].

Rat endometrial stromal cells express cx43 during decidualization in vitro (Fig. 3); this also occurs during in vivo decidualization in the rat (Fig. 2) and in vitro decidualization of human endometrial stromal cells [11]. In addition, during in vitro decidualization, cx26 transcripts (Fig. 3) were expressed; but in contrast to observations during in vivo decidualization, cx26 steady-state levels rapidly declined. There are at least four possible explanations for this decline during decidualization in vitro: 1) the endometrial stromal cell cultures were initially contaminated with epithelial cells; 2) our culture system selects for decidual cells that resemble those found in the deeper parts of the antimesometrium; 3) cx26 requires hormones to maintain its expression; 4) it is an artifact of culturing the cells.

Since luminal epithelial cells express cx26 [14], contamination of the cultures with luminal epithelial cells would result in detection of cx26 transcripts during in vitro decidualization. This is unlikely to have occurred, because cytokeratin staining, an indicator of epithelial cells, was at background levels at 2 h as evidenced by immunohistochernistry (results not shown).

The decline in cx26 transcript steady-state levels during in vitro decidualization could be the result of conditions that select for a subset of decidual cells that are usually located in the deeper decidua. Winterhager and others [14] reported that cx26 was found in the stromal cells that lie in close proximity to the luminal epithelium whereas cx43 was found in the deeper decidua.

Alternatively, the absence of steroid hormones in the culture medium may have resulted in the decline of cx26 steady-state mRNA levels. Administration of estradiol to ovariectomized [15] and immature animals [36] increased cx26 steady-state mRNA levels in endometrial scrapings and whole uteri, respectively. Immunohistochemistry indicated that the increase in cx26 staining was confined to the luminal epithelium, presumably suggesting that estradiol modulates cx26 expression only in the epithelium. By contrast, progesterone did not affect cx26 steady-state levels in the stroma under these circumstances [15, 36]. Thus it is unlikely that steroids modulate cx26 expression in endometrial stromal cells.

Finally, the decline in cx26 steady-state levels during in vitro decidualization may be the consequence of an in vitro artifact. At present we do not know why cx26 steady-state levels decline, but possible mechanisms are currently being studied.

In the immunofluorescence studies, we found punctate immunofluorescent signals around the periphery of the cells, suggesting that cx43 is assembled into membrane plaques (Fig. 5). In addition, by comparison with the preimmune serum, the antibody gave diffuse signals presumably located within the cells, suggesting that cx43 may also be located intracellularly. These results suggest that cx43 is present in the cells at 40 h in culture, and provide further evidence that gap junctions form between endometrial stromal cells during culture.

To determine whether functional gap junctions form between endometrial stromal cells during culture, we applied two dye-coupling techniques: preloading and scrape-loading. In the preloading experiments, preloaded cells could be identified because of the presence of fluorescence due to both DiI and calcein. When fluorescence attributable to calcein but not DiI was found adjacent to a preloaded cell, this was taken as evidence for the presence of gap junctions between the cells. When both DiI and calcein fluorescence were found in adjacent cells, this was interpreted either as two preloaded cells having plated together or as the existence of cytoplasmic bridges between the cells [29, 30]. By these criteria, dye coupling consistent with the presence of gap junctions between the cells was found in the endometrial stromal cells as early as 3 h in culture (Fig. 6). Statistical analysis of the degree of coupling in the preloading experiments suggested that the extent of dye coupling increased from 3 to 6 h in culture.

Scrape-loading allowed us to determine the ability of the cells to dye-couple at later times in the culture period. For scrape-loading, the transfer of CF but not RD to contiguous cells from the scrape line suggests that gap junctions, not cytoplasmic bridges, are present between the cells [31]. The selective transfer of CF was found at 24, 48, and 72 h in culture (Fig. 7). Because scrape-loading is a qualitative method, quantitative comparisons were not made [37]. The dye-coupling experiments suggest that endometrial stromal cells develop functional gap junctions early during culture (3 h) and that they remain present throughout the culture period (up to 72 h). The interpretation of the dye-coupling experiments is limited because they were conducted on cells in culture, but these experiments do provide evidence that the decidual gap junctions formed in vitro are functional. If, as in vitro, decidual gap junctions are functional in vivo, then their role may be to allow the coordination of growth and subsequent regression of the antimesometrial decidua [5, 8].

In conclusion, the patterns of cx26 and cx43 transcript steady-state levels are similar during artificially induced decidualization and pregnancy. Both cx26 and 43 transcript steady-state levels increased during artificially induced decidualization. During decidualization in vitro, cx26 transcript steady-state levels decreased whereas those for cX43 increased. Finally, the evidence strongly suggests that decidual gap junctions are functional. These data support the original hypothesis by Finn and Lawn [3] that "specialized junctions" might form a functional syncytium in the endometrium to coordinate decidualization.

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REFERENCES

1. Weitlauf HM. The biology of implantation. In: Knobil E, Neill JD (eds.), The Physiology of Reproduction, vol. 1, 2nd ed. New York: Raven Press Ltd.; 1994: 391-440.
2. Ono H, Ide C, Nishiya I. Electron microscopic study on early decidualization. of the endometrium of pregnant mice, with special reference to gap junctions. Placenta 1989; 10:247-261.
3. Finn CA, Lawn AM. Specialized junctions between decidual cells in the uterus of the pregnant mouse. J Ultrastruct Res 1967; 20:321-327.
4. Parkening T. An ultrastructural study of implantation in the golden hamster. III. Initial formation and differentiation of decidual cells. J Anat 1976; 122:485 -498.
5. Kleinfeld RG, Morrow HA, DeFeo VJ. Intercellular junction between decidual cells in the growing deciduoma of the pseudopregnant rat uterus. Biol Reprod 1976; 15:593603.
6. O'Shea JD, Kleinfeld RG, Morrow HA. Utrastructure of decidualization in the pseudopregnant rat. Am J Anat 1983; 166:271- 298.
7. Brökelman J, Biggers JD. Studies on the development of cell contacts and of the inter cellular matrix during decidualization in the rat. Arch Gynecol 1979; 227:103-117.
8. Parr MB, Tung HN, Parr EL. The ultrastructure of the rat primary decidual zone. Am J Anat 1986; 176:423-436.
9. Lawn AM, Wilson EW, Finn CA. The ultrastructure of human decidual and predecidual cells. J Reprod Fertil 1971; 26:85-90.
10. Irwin JC, Kirk D, King RJB, Quigley MM, Gwatkin RB. Hormonal regulation of human endometrial cells in culture: an in vitro model for decidualization. Fertil Steril 1989; 52:761-768.
11. Semer D, Reisler K, MacDonald PC, Casey ML. Responsiveness of human endometrial cells to cytokines. Ann NY Acad Sci 1991; 622:99-110.
12. Beyer EC, Goodenough DA, Paul DL. The connexins, a family of related gap junction proteins. In: Hertzberg EL, Johnson RG (eds.), Gap junctions. New York: Alan R, Liss, Inc.; 1988:167-175.
13. Winterhager E, Stutenkemper R, Traub 0, Beyer E, Willecke K. Expression of different connexin genes in rat uterus during decidualization and at term. Eur J Cell Biol 1991; 55:133-142.
14. Winterhager E, Grümmer R, Jahn E, Willecke K, Traub O. Spatial and temporal expression of connexin26 and connexin43 in rat endometrium during trophoblast invasion. Dev Biol 1993; 157:399-409.
15. Grümmer R, Chwalisz K, Mulholland J, Traub O, Winterhager E. Regulation of connexin26 and connexin43 expression in rat endometrium by ovarian steroid hormones. Biol Reprod 1994; 51:1109-1116.
16. Winterhager E, Brümmer F, Dermietzel R, Hülser DF, Denker H-W. Gap junction formation in rabbit uterine epithelium in response to embryo recognition. Dev Biol 1988; 126:203-211.
17. Kennedy TG, Ross HE. Effect of prostaglandin E_2 on rate of decidualization in rats. Prostaglandins 1993; 6:243-250.
18. McCormick SA, Glasser SR. Differential response of individual uterine cell types from immature rats treated with estradiol. Endocrinology 1980; 106:1634-1649.
19. Yee GM, Kennedy TG. Prostaglandin E_2, cAMP, and cAMP-dependent protein kinase isozymes during decidualization of rat endometrial stromal cells in vitro. Prostaglandins 1993; 46:117-138.
20. Bitton-Casimiri V, Rath NC, Psychoyos A. A simple method for separation and culture of rat uterine epithelial cells. J Endocrinol 1977; 73:537-538.
21. Martel D, Psychoyos A. Endometrial content of nuclear estrogen receptor and receptivity for ovoimplantation in the rat. Endocrinology 1976; 99:470-475.
22. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987; 162:156-159.
23. Sambrook J, Fritsch EF, Maniatis T. Molecular Cloning. A Laboratory Manual, 2nd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 1989.
24. Zhang JT, Nicholson BJ. Sequence and tissue distribution of a second protein of hepatic gap junctions, cx26, as deduced from its cDNA. J Cell Biol 1989; 109:3391-3401.
25. Beyer EC, Paul DL, Goodenough DA. Connexin43: a protein from rat heart homologous to a gap junction protein from the liver. J Cell Biol 1987; 105:2621-2629.
26. Smith CL, Hammond GL. Ontogeny of corticosteroid-binding globulin biosynthesis in the rat. Endocrinology 1991; 128:983-988
27. DeSousa PA, Valdimarsson G, Nicholson BJ, Kidder GM. Connexin trafficking and the control of gap junction assembly in mouse preimplantation embryos. Development 1993; 117:1355-1367.
28. Laird DW, Puranam KL, Revel JP. Turnover and phosphorylation dynamics of connexin43 gap junction protein in cultured cardiac myocytes. Biochem J 1991; 276:67-72.
29. Tomasetto C, Neveu MJ, Daley J, Horan PK, Sager R. Specificity of gap junction communication among human mammary cells and connexin transfectants in culture. J Cell Biol 1993; 122:157-167.

30. Goldberg GS, Bechberger JF, Naus CCG. A pre-loading method of evaluating gap junctional communication by fluorescent dye transfer. Biotechniques 1995; 18:490-497.
31. el Fouly MH, Trosko JE, Chang C. Scrape-loading and dye transfer: a rapid and simple technique to study gap junctional intercellular communication. Exp Cell Res 1987; 168:422-430.
32. Snedcor GW. Statistical Methods, 5th ed. Ames, IA: Iowa State University Press; 1956.
33. Steele RGD, Torrie JH. Principles and Procedures in Statistics. New York: McGraw Hill; 1960.
34. Garfield RE, Kannan MS, Daniel EE. Gap junction formation in myometrium: control by estrogens, progesterone, and prostaglandins. Am J Physiol 1980; 238:C81-C89.
35. Yee GM, Squires PM, Cejic SS, Kennedy TG. Lipid mediators of implantation and decidualization. J Lipid Mediat 1993; 6:525-534.
36. Risek B, Wier FG, Phillips A, Habit DW, Gilula NB. Gap junction regulation in the uterus and ovaries of immature rats by estrogen and progesterone. j Cell Sci 1995; 108:10171032.
37. McKarns SC, Doolittle DJ. Limitations of the scrape-loading/dye transfer technique to quantify inhibition of gap junctional intercellular communication. Cell Biol Toxicol 1992; 8:89-103.