Presence and localization of connexins 43 and 26 in cell cultures derived from myometrial tissues from nonpregnant and pregnant women and from leiomyomas

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OBJECTIVE: Our objective was to study the appearance and distribution of connexins 43 and 26 in various human myometrial cell cultures.

STUDY DESIGN: Scrape loading, Western blotting, and immunohistochemical techniques were applied to cultured cells derived from myometrial tissues obtained from nonpregnant and pregnant women (upper and lower uterine segments) and from leiomyomas (tumor and analogous myometrial tissues).

RESULTS: Scrape loading revealed the presence of metabolic coupling in all tissues. Indirect immunohistochemical studies showed membrane localization of connexin 43 in all myometrial cultures. Western blots and indirect immunohistochemical studies showed the presence and localization of the connexin 26 protein and associated gap junctions in tissues from myomas and from nonpregnant and pregnant women except for those derived from the upper segment of the pregnant uterus.

CONCLUSION: These results show that human myometrial cultures express various gap junction proteins and that there are regional differences in expression of connexins in tissues from pregnant women. (Am J Obstet Gynecol 2000;182:926-30.)

Key words: Gap junctions, myometrium, connexin

The onset of labor is associated with several events that include the appearance of gap junctions between the smooth muscle cells in the myometrium.[1] Gap junctions are made up from a family of proteins, called connexins, that can be classified according to their molecular weights.[2]

It has been shown that at least three members of the family of connexins (Cx43, Cx45, and Cx26) are associated with various stages of pregnancy in rat myometrium.[3, 4] The appearance of Cx43 in the myometrium coincides with the onset of labor in several species, including women.[5] The presence of the Cx43 protein and immunohistochemically detectable gap junctions have also been shown in some human leiomyomas from premenopausal nonpregnant women but not in the autologous myometrium.[6] The timing of expression and appearance of Cx43 shows similarities between rats[7] and human subjects,[8] but the existence of a variety of connexin expressions has not been shown in the latter.

The aim of our study was to examine the presence of Cx43 and Cx26 proteins and the distribution of gap junctions in cell cultures prepared from myometrial tissues obtained from the upper and lower uterine segments of nonpregnant and pregnant women and from leiomyoma tissues.

Material and methods

Cell culture. Strips of myometrium from nonpregnant women and myoma tissue were obtained at hysterectomy and from the upper and lower uterine segments of the pregnant uterus at cesarean delivery. Tissues obtained from the myomas were prepared separately: one tissue specimen dissected from the tumor tissue itself and the other, from the analogous myometrium. The specimen from the tumor tissue was taken 1 cm below the surface of the myoma.

The protocol of the study was approved by the institution's ethics committee, and the patients were informed and gave consent to participate in the study. The tissues were minced into very small pieces that were rinsed with phosphate-buffered sodium choride solution (PBS) repeatedly and then digested overnight with collagenase 2.5 mg/mL (Sigma Chemical Co, St Louis, Mo), hyal-

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uronidase 200 g/mL (Life Technologies, Inc, Grand Island, NY), and deoxyribonuclease 50 g/mL (Life Technologies) at 37°C in Dulbecco modified Eagle medium (Life Technologies). The cells were cultured in the Dulbecco medium supplemented with 5% fetal bovine serum (Life Technologies), penicillin G 100 IU/mL (Gibco), and streptomycin sulfate 50 g/mL (Life Technologies) in multiwell dishes. Incubation was carried out in 5% carbon dioxide at 37°C. The viability of the cells was >85%, as assessed by trypan blue dye exclusion.

Scrape loading. Scrape loading was used to study metabolic coupling qualitatively between myometrial cells, as described by El-Fouly et al.[9] Briefly, the cells were rinsed with PBS, and 0.05% lucifer yellow CH in PBS was added. The bottom of the well was scraped with a razor blade, and the dye was left for 2 minutes. Then the cells were rinsed with PBS and the culture medium was added. Dye transfer was examined under a fluorescent microscope, and photographs were taken.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blot analysis. All samples were concentrated about 5-fold in prerinsed Nanosep 10 k (Pall-Filtron; Pall Medical, Ann Arbor, Mich) centrifugal concentrators and then diluted in loading buffer (10-mmol/L Tris hydrochloride, 1-mmol/L ethylenediaminetetraacetic acid, 2.5% sodium dodecyl sulfate [wt/vol], and 5% β-mercaptoethanol [vol/vol]; pH 8.0).

Protein separation for silver staining and Western blot analysis were performed on a Phast (Amersham Pharmacia Biotech, Uppsala, Sweden) system by means of two precast 8% to 25% acrylamide gradient gels, running in parallel for 80 ampere volt hours. One of the gels contained biotin-conjugated molecular weight markers (Amersham Pharmacia Biotech). Marker proteins were treated as described here before loading.

One of the gels was silver stained according to the manufacturer's instructions (Pharmacia Development Technique File 210). The other gel was electroblotted onto Immobilon P (Millipore Corp, Bedford, Mass) blotting membrane by means of semidry transfer, according to Pharmacia Development Technique File 221. Transfer buffer consisted of 0.7-mol/L glycine and 25-mmol/L Tris hydroxyacetate (pH 7.7), and blots were performed for 30 minutes at 25 mA. All incubations of blotting membrane were carried out at 37°C under gentle agitation for 30 to 60 minutes. Nonspecific binding was blocked in 3% [wt/vol] bovine serum albumin (Cohn fraction V, Sigma) in 25-mmol/L Tris hydrochloride and 140-mmol/L sodium chloride (pH 8.0; Tris-buffered saline solution) and was subsequently treated with polyclonal rabbit antisera raised against Cx26 (Zymed Corp, San Francisco) to a final dilution of 1:2000 in 20-mmol/L phosphate, 140-mmol/L sodium chloride, and 0.2% [vol/vol] Triton X-100. The secondary antibody was donkey antirabbit horseradish-peroxidase, conjugated (Amersham Pharmacia Biotech) to a final dilution of 1:1000 [wt/vol] of bovine serum albumin in Tris-buffered saline solution. Streptavidin-horseradish-peroxidase conjugate 1:1000 [wt/vol] (Amersham Pharmacia Biotech) was added for the detection of molecular weight markers. All rinses between incubations were done with Tris-buffered saline solution containing 0.1% [vol/vol] Tween-20. Blots were developed with the Enhanced Chemiluminescence Kit (Amersham Pharmacia Biotech) according to the manufacturer's instructions. For documentation, a Biomax (Eastman Kodak, Rochester, NY) film was used.

Indirect immunofluorescence. The cells were rinsed with PBS (pH 7.4) at 37°C and then fixed with 4% buffered formalin for 15 minutes at room temperature and in methanol for 5 minutes at –20°C. The cells were washed with PBS and incubated in blocking solution (2% human serum albumin, 0.2% Triton X-100 in PBS) for 15 minutes. They were then stained with either an anti-Cx43 mouse monoclonal antibody (Zymed Laboratories) 1:200 diluted in 0.2% Triton X-100 in PBS or an anti-Cx26 rabbit polyclonal antibody (Zymed), 1:100 diluted in 0.2% Triton X-100 in PBS. Incubations were carried out for 30 minutes at room temperature. After being rinsed, the cells were incubated with a secondary antibody, fluorescein isothiocyanate–conjugated rabbit antimouse antibody (Serotec Ltd, Oxford, United Kingdom) 1:200 in PBS and tetramethylrhodamine isothiocyanate (Zymed). Negative controls were cultured cells that were incubated according to the protocol described here but with an irrelevant immunoglobulin $G_{2\alpha}$ as primary antibody. Nonspecific staining was not detected. Examination was then conducted with an inverted Nikon Diaphot microscope equipped with a reflected light fluorescence system for application of fluorescein isothiocyanate, and photographs were taken (Tmax 400 ASA, Eastman Kodak).

Results

Scrape loading illuminated the layer of cells at the immediate vicinity of the scraped tissue in all cultures. The adjacent cells (second layer) were also illuminated, and the intensity of fluorescence was diminished gradually on the axis perpendicular to the scrape line (Fig 1).

Western blot analysis showed that an immunopositive band with an apparent molecular weight of approximately 23 kd could be detected in tissues obtained from leiomyoma, analogous myometrium, and myometrium of the lower uterine segment from both pregnant and nonpregnant women, but it could not be detected in the upper segment of the myometrium from pregnant women (Fig 2). The negative staining in the upper segment did not depend on low total protein concentration, which was confirmed by the silver-stained gel (data not shown).

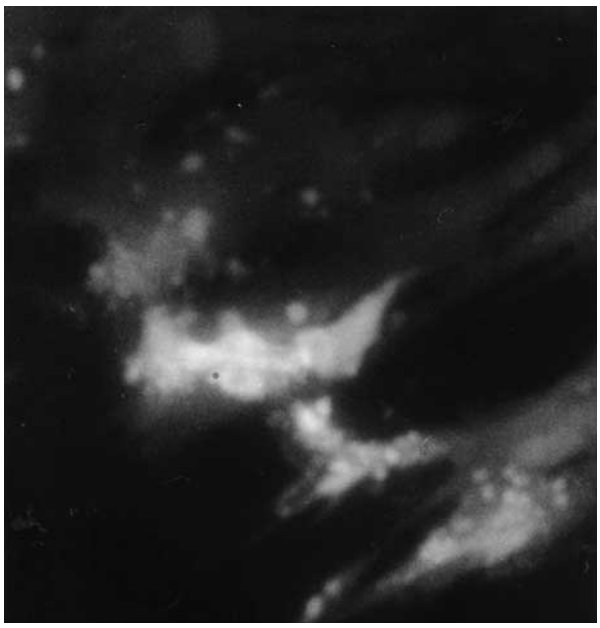

Fig 1. Scrape-loading photomicrograph of human myometrial cultures. Illumination of cells adjacent to scrape line (*lower right corner*) is seen. Intensity of fluorescence gradually decreases away from first layer of cells. Axis of propagation is from *lower left* to *upper right* corner of figure.

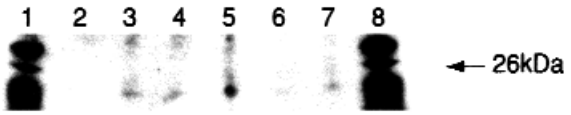

Fig 2. Western blots of Cx26 from various human myometrial cultures. Contrast of image is enhanced. *Left to right*, Molecular weight marker, upper segment—pregnancy, lower segment—pregnancy, myoma, lower segment—nonpregnancy, analogous myometrium, upper segment—nonpregnancy, and molecular weight marker. Lack of band in upper segment of myometrium from pregnancy is seen.

The characteristic pattern of punctual staining by Cx43 was observed in all cultures (Fig 3, *a* to *e*). The localization of the fluorescent spots was in accordance with the plasma membrane. The pattern and intensity of Cx43 staining varied slightly between the cultures; tissues from the upper segment of the pregnant uterus showed longitudinal and intense staining (Fig 3, *c*); nonpregnant, lower-segment, and analogous myometrial tissues did not display any particular pattern, and the frequency of spots was lower than in the upper segment (Fig 3, *a, b,* and *e*); and myoma tissues showed a wheatfield-like appearance and intense staining (Fig 3, *d*). When the cultures were stained by Cx26 antibody, fluorescent spots were detected in line with the plasma membrane of the cells, as in Cx43, except from those obtained from the upper segment of the myometrium from pregnancy, in which no staining could be detected (Fig 3, *h*). The staining intensity of Cx26 was weaker than that of Cx43 in all cultivated tissues (Fig 3, *f, g, i,* and *j*).

Comment

The cultivated myometrial tissues in our study exhibit (1) metabolic coupling as shown by scrape loading, (2) Cx26 protein in all tissues except in myometrium from the upper uterine segment in pregnant women, and (3) immunohistochemically detectable Cx43 and Cx26 gap junctions between the cells of pregnant, nonpregnant, myomatous, and analogous myometrium, except for Cx26, which was not present in the tissues from the upper uterine segment in pregnancy. Therefore these results indicate that (1) functional coupling of cultivated human myometrial cells is established through gap junctions made up of at least 2 different connexin proteins and (2) the presence and appearance of connexins display regional differences in human myometrium in pregnancy.

It has previously been shown by electron microscopy[10] and electrophysiologic examination[11-14] that the frequency of gap junctions increases significantly during labor. The expression of Cx43 is also increased with the onset of labor[8] and is accompanied by the appearance of immunohistochemically detectable Cx43 gap junctions in the myometrium.[5, 15] Recently, patch-clamp experiments showed that Cx43 is the major gap junction protein in the human myometrium.[16] Taken together, these findings indicate that Cx43 gap junctions may contribute to the synchronization of uterine contractions during labor by establishing an extensive intercellular communication between myometrial cells. The extensive presence of Cx26 protein and gap junctions among cultivated myometrial tissues in our study indicates that gap junctional intercellular communication may not be solely responsible for a functionally syncytial tissue in labor. It was previously shown in myometrium from pregnant rats that temporal expressions of Cx43 and Cx26 differed: Cx26 was elevated before labor, whereas Cx43 expression peaked on the day of labor.[4] The regulation of expression also varied; Cx26 was up-regulated by progesterone[4] and Cx43 was up-regulated by estradiol.[17] Another gap junction protein, Cx45, which has not yet been identified in human subjects, was expressed at early gestation and in postpartum rat myometrial tissues.[3] It is likely, when the myometrium is in quiescence, that intercellular communication is established by gap junctions formed by several members of the connexin family, but the need to enhance the coupling of the cells during labor is maintained by an increase in Cx43 expression and subsequent gap junction formation. We previously showed the presence of metabolic[18] and electrical[19, 20] coupling between human myometrial cells before labor. It has been reported that when the cytoplasmic tail of Cx43 in human cardiac gap junctions was shortened with site-directed

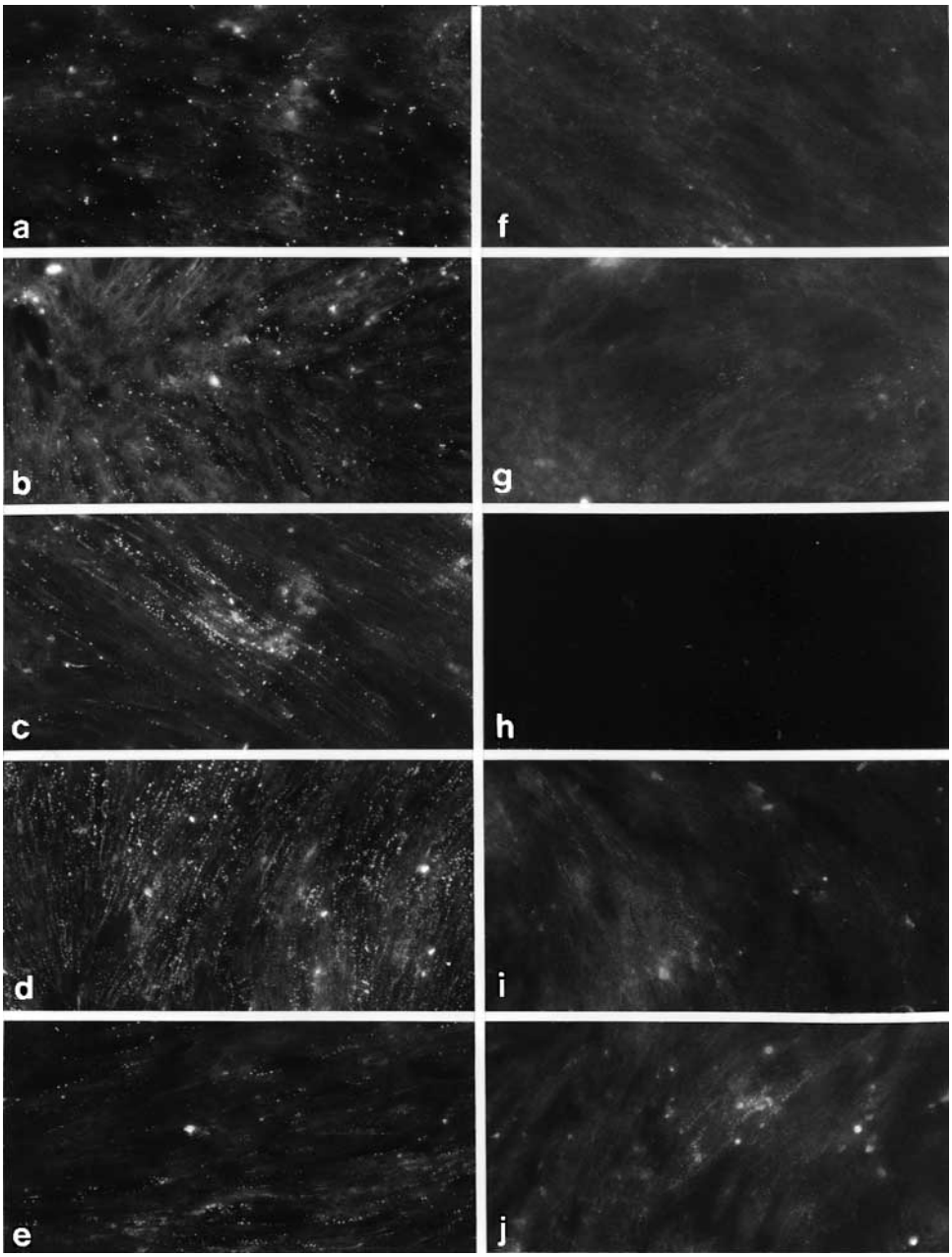

Fig 3. Indirect immunohistochemical results of human myometrial cultures with Cx43 (**a-e**) and Cx26 (**f-j**). Cultures obtained from myometrium of nonpregnancy (**a** and **f**), from *lower segment* (**b** and **g**) and *upper segment* (**c** and **h**) of myometrium during pregnancy, from myoma (**d** and **i**), and from analogous tissue (**e** and **j**) are seen.

mutagenesis to the length of Cx26 the unitary conductance of the channels was decreased to 50 ps from 60 and 100 ps.[21] Our previous results on studies of pregnant human myometrium before labor showed that dye coupling was more extensive when a fluorescent probe with a smaller molecular mass was injected intracellularly than when a probe with a higher mass was used.[18] If the lower conductance of Cx26 gap junction channels is caused by a smaller pore size, then the observed difference between fluorescent probes in metabolic coupling in nonlabor human tissues may be a result of the dominance of Cx26 gap junctions. This hypothesis can be confirmed indirectly by quantitative analysis of Cx26 protein and immunohistochemically detectable gap junctions in these tissues.

The absence of Cx26 protein and gap junctions in cultures obtained from the upper segment of myometrium during pregnancy indicates regional differences in the connexin expression in human myometrium. We observed the presence of Cx26 gap junctions when we ob-

tained biopsy specimens from regions that were close to the upper segment in myometrium from nonpregnant subjects. However, these segments are not clear, as in the tissue from pregnancy, and it is likely that the enlargement of the myometrium during pregnancy is accompanied by regional variances in protein expression so that functional activity can be maintained during labor.

Our study is, to our knowledge, the first to demonstrate the presence of Cx26 protein and gap junctions in both the tumor tissue and the analogous myometrium. The presence of Cx43 gap junctions was previously shown in leiomyomas, but they were absent in the analogous tissue when the tissue was devoid of 17β-estradiol.[6] Our results are in contrast to the latter finding, and the difference may arise in preparation and maintenance of the tissue.

In future studies researchers need to clarify the temporal expression and regulation of Cx26 protein so that the significance of various gap junction proteins in human myometrium during pregnancy and in myomas can be understood.

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