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Temporal and spatial expression of AIF in the mouse uterus during early pregnancy

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1. ABSTRACT

Apoptosis-inducing factor (AIF) is a phylogenetically old, bifunctional protein with a pro-apoptotic function and redox activity. AIF regulates apoptosis and also plays a role in the defense against stress depending on its subcellular localization. Embryo implantation is a complicated process, in which an activated blastocyst interacts with a receptive uterus. The expression and regulation of AIF were investigated in this study in the mouse uterus during early pregnancy, pseudopregnancy, delayed implantation, artificial decidualization and under hormonal treatment using *in situ* hybridization, immunohistochemistry and real-time PCR. During early pregnancy, temporally and spatially regulated patterns of AIF expression were found in the mouse uterus; AIF expression in the luminal epithelium and glandular epithelium is regulated by steroid hormones; AIF mRNA expression in the stroma is influenced by the active blastocyst; and AIF protein was found to be located in the cytoplasm rather than the nucleus through confocal microscope. Our data suggest that AIF might play an important role during mouse embryo implantation and that the role of AIF might be implemented through its physiological activity rather than through its pro-apoptotic function in the mouse uterus during this period.

2. INTRODUCTION

AIF is a bifunctional protein with both pro-apoptotic and pro-survival functions. AIF plays a vital role in the maintenance of mitochondrial and energy metabolism. Based on findings from various knockout/knockdown models, AIF has been found to play a role in oxidative phosphorylation, mainly by modulating the structure and function of complex I of the respiratory chain (1, 2). AIF functions as a FAD-containing NADH-dependent oxidase (3). Additionally, following an apoptotic stimulus, AIF translocates into the nucleus and triggers chromatin condensation and large-scale DNA fragmentation, thus inducing cell death. AIF was discovered as the first caspase-independent death effector.

AIF is encoded by a single gene located on the X chromosome from which a 67-kDa precursor molecule is synthesized in the cytosol (4). When AIF is imported into mitochondria because of a mitochondrial leading sequence, an N-terminal 52-amino-acid sequence is cleaved off in the matrix by the mitochondrial processing peptidase to form an inner membrane-anchored mature form (62 kDa). During apoptosis, the protein is further proteolytically processed at amino acid position 101/102 in the

intermembrane space and detaches from the inner membrane, followed by permeabilization of the outer membrane and is released into the cytoplasm, thus forming mature AIF (57 kDa) (5). Then, mature AIF is transported into the nucleus from the cytoplasm via its nuclear leading sequence to induce cell death.

AIF is not a universal cell death effector, and its contribution to carrying out the cell death process is dependent not only on cell type but also on the apoptotic insult. Genotoxic agents, exitotoxicity induced by glutamate, hypoxia–ischemia followed by reperfusion, neurodegeneration and pathogen exposure are all associated with apoptosis induced by AIF (6). Cytoplasmic Hsp 70 and Akt interact with AIF and inhibit its relocation to the nucleus (7, 8).

Embryo implantation is a mutual interaction between the blastocyst and the uterus. Successful implantation is dependent on the cellular and molecular dialogue between competent embryos and a receptive uterus (4). Many signals, such as hormones and cytokines, are temporally expressed and participate in this complex process (9, 10), and some factors are strictly regulated by two sex steroid hormones, progesterone (P4) and 17-beta estradiol (E2) (11). Many biological events become linked to fundamental cellular pathways of growth, differentiation and apoptosis during this period. In response to an implanting conceptus, endometrial fibroblast-like cells differentiate into large decidual cells. This re-differentiation of cells occurs in a temporally and regionally regulated fashion. It first begins just after the onset of implantation in a small number of cells adjacent to the implanting conceptus in the antimesometrial region of the endometrium, forming what is referred to as the primary decidual zone (PDZ). This process is followed by the progression of decidualization toward the myometrium in the antimesometrial region of the uterus, which forms the secondary decidual zone (SDZ) (12). As a bifunctional protein involved in both cell survival and cell death, AIF might be associated with this process. However, the expression, regulation and function of AIF in the mouse uterus during embryo implantation have yet to be determined. In this study, we investigated the expression and regulation of AIF in the mouse uterus to further define its function during the peri-implantation period.

3. MATERIALS AND METHODS

3.1. Animals and treatments

Mature mice (ICR) were caged in a controlled environment with a 14 h light: 10 h dark cycle. All animal procedures were approved by the Institutional Animal Care and Use Committee of Northeast Forestry University. Female mice were mated with fertile or vasectomized males of the same strain to induce pregnancy or pseudopregnancy (day 1 is the vaginal plug day). On days 1 to 4, pregnancy was confirmed by flushing embryos from the oviducts or uterus. Implantation sites were identified on day 5 by intravenous injection of 0.1 ml of 1% Chicago blue dye (Sigma, St. Louis, MO) in saline.

To induce delayed implantation, pregnant mice were ovariectomized under ether anesthesia at 0830 h-0900 h on day 4 of pregnancy. Delayed implantation was maintained by daily s.c. injection of P4 (1 mg per mouse, Sigma) on days 5 to 7. To terminate delayed implantation, progesterone-primed delayed-implantation mice were treated with E2 (25 ng per mouse, s.c., Sigma) on day 7. The mice were sacrificed to collect their uteri 24 h after estrogen treatment. Delayed implantation was confirmed by flushing blastocysts from one horn of the uterus, and the activation of delayed implantation was identified by intravenous injection of Chicago blue dye solution.

Artificial decidualization was induced by intraluminally infusing 0.25 mL of sesame oil into one uterine horn on day 4 of pseudopregnancy, and the contralateral uninjected horn served as a control. The uteri were collected on day 8 of pseudopregnancy. Decidualization was confirmed by weighing the uterine horn (13).

Hormonal treatments were initiated two weeks after mature female mice were ovariectomized. The ovariectomized mice were treated with an injection of E2 (100 ng per mouse, Sigma), P4 (1 mg per mouse, Sigma) or a combination of these doses of P4 and E2. All steroids were dissolved in sesame oil and injected s.c. Treated mice were killed 2 h, 6 h, 12 h or 24 h later. Controls received only the vehicle (0.1 ml per mouse).

3.2. Real-time PCR

Total RNAs from uteri were isolated using TRIZOL reagent (Takara, D9108 A) according to the manufacturer's instructions. cDNA was reverse transcribed from 1 μg of total RNA using the PrimeScript® RT reagent Kit With gDNA Eraser (Perfect Real Time) (Takara, DRR047 S). For real-time PCR, cDNA was amplified using SYBR® Premix Ex TaqTM (Takara, DRR041 A) according to the manufacturer's instructions. PCR was performed with a Real-time PCR System (ABI PRISM 7500 Real-time PCR System, Applied Biosystems, CA) with the following conditions: 94°C 30 sec, 55°C 5 sec, 60°C 34 sec for 40 cycles. The RT-PCR conditions were optimized, and the melting curves presented a single amplified band. The primers were as follows: AIF (NM_012019) forward 5`-GGCTTCCGGGTAAATGCAGA-3`and reverse 5`-TCGCCTTCGACCCAACTTTATATC-3` (96 bp), which were designed by TaKaRa; GAPDH (NM_001001303) forward 5`-AAATGGTGAAGGTCGGTGTG-3`and reverse 5`-TGAAGGGGTCGTTGATGG-3` (108 bp), which were used as described previously (14).

3.3. Hybridization probes

Total RNA from the mouse uterus was reverse-transcribed and amplified with the corresponding primers. The amplified fragment of each gene was cloned into the pGEM-T plasmid (pGEM-T Vector System 1, Promega, Madison, WI) and verified by sequencing. The recombinant plasmid was amplified with primers for T7 and SP6 to prepare templates for labeling sense or antisense probes.

Digoxigenin (DIG)-labeled antisense or sense cRNA probes were transcribed *in vitro* using a DIG RNA labeling kit (Roche Diagnostics GmbH, Mannheim, Germany).

3.4. *In situ* hybridization

Uteri were sectioned into 4- to 6-mm pieces and flash-frozen in liquid nitrogen. Frozen sections (8 μm) were mounted on 3-aminopropyltriethoxy-silane (Sigma)–coated slides and fixed in a 4% paraformaldehyde solution in PBS. The sections were washed in PBS twice, treated in 1% Triton X-100 for 20 minutes, and washed in PBS three times. After prehybridization in a solution of 50% formamide and 5X SSC (1X SSC is 0.15 M sodium chloride and 0.015 M sodium citrate) at room temperature for 15 minutes, the sections were hybridized in hybridization buffer (5X SSC, 50% formamide, 0.02% bovine serum albumin, 250 mg/mL of yeast transfer RNA, 10% dextran sulfate, 1 mg/mL of denatured digoxigenin-labeled antisense RNA probes for mouse AIF) at 55ºC for 24 hours. After hybridization, the sections were washed in 50% formamide-5X SSC at 55ºC for 15 minutes, 50% formamide-2X SSC at 55ºC in a rocking bed for 30 minutes, in 50% formamide-0.2X SSC at 55ºC twice for 30 minutes each and in 0.2X SSC at room temperature for 5 minutes. The sections were incubated with a sheep anti-digoxigenin antibody conjugated to alkaline phosphatase (1:5, 000; Roche) in 0.5% blocking solution overnight at 4ºC after nonspecific binding was blocked in 1% blocking solution for 1 hour. Signals were visualized with 0.4 mM 5-bromo-4-chloro-3-indolyl phosphate and 0.4 mM nitroblue tetrazolium in buffer containing 100 mM Tris-HCl (pH 9.5), 100 mM NaCl, and 50 mM $MgCl_2$. Endogenous alkaline phosphatase activity was inhibited with 2 mM levamisole (Sigma). All of the sections were counterstained with 1% methyl green. Positive signals were visualized as a dark-brown color.

3.5. Immunohistochemistry

A polyclonal antibody against mouse AIF that can be used for detection of all three forms of AIF (67 kDa, 62 kDa and 57 kDa) (D-20, cat. n◦ SC-9416, Santa Cruz, USA) was diluted 1:1000 for immunohistochemistry (15). Frozen uteri were sectioned at thicknesses of 8 μm, mounted on slides, and then fixed in 4% formaldehyde in PBS for 20 minutes. Sections were washed in PBS for 5 min three times and then incubated in a blocking solution containing 10% normal horse serum for 30 min before incubation in primary antibody overnight at 4ºC. Immunostaining was performed using a rabbit anti-goat IgG-FITC antibody (FSA 0005, MAI BIO CO. LTD, China) at 37ºC for 1 h. After being washed in PBS three times for 5 min each, the nuclei were counterstained with the DNA-specific dye 4`, 6`-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) for 5 min at room temperature. Positive signals were visualized as green fluorescence, and the nucleus was visualized based on blue fluorescence under a microscope (Carl Zeiss, Germany) equipped with a cooled three-chip charge-coupled-device camera (Perkin Elmer Ultraview Scanning Disc Microscope). Moreover, the same concentration of normal horse IgG was used in place of the corresponding primary antibody as a negative control.

4. MICROSCOPIC ASSESSMENT AND STATISTICAL ANALYSIS

Uterine samples were analyzed from three mice in each treatment group. Experiments were repeated at least three times, from which a result representative of at least three similar results was presented for the *in situ* hybridization and immunohistochemical analyses. Data from all three groups were presented for the real-time PCR. The signal intensities of AIF detected by *in situ* hybridization and immunohistochemistry were assessed by microscope. Triplicate cycle threshold Ct values were analyzed with Microsoft Excel using the comparative Ct (2-delta delta Ct) method (16). The means of the measured values were used to obtain the ratio between AIF mRNA and GAPDH mRNA to represent the relative expression level of AIF mRNA as determined by real-time quantitative PCR analysis.

5. RESULTS

5.1. AIF expression in the mouse uterus during early pregnancy

Real-time quantitative PCR analysis (see Figure 1A for the melting curve for AIF mRNA) showed the relative expression level of AIF mRNA in the uterus from days 1 to 8 of pregnancy. Compared to that of day 1 pregnant mice, the level of AIF mRNA in the mouse uterus gradually increased from days 2 to 4 of pregnancy. It was then sharply lower on day 5 of pregnancy, even lower than that on day 2. The level of AIF mRNA showed a tendency to be up-regulated from days 5 to 7 of pregnancy, and then was slightly lower on day 8 (Figure 1B).

The *in situ* hybridization results showed that there was a basal level of AIF mRNA expression in the luminal epithelium (LE) and glandular epithelium (GE) on day 1 of pregnancy (Figure 2D1). The signal in the LE and GE from days 2 to 4 of pregnancy was higher (Figure 2D2, D3, D4). On day 5 of pregnancy, AIF mRNA was not detected in the LE but was detected in the stromal cells surrounding the embryo (Figure 2D5m). There was a low level of expression in the LE at inter-implantation sites (Figure 2D5n). From days 6 to 8 of pregnancy, the signal was strongly confined to the SDZ and gradually increased (Figure 2D6m, D7, D8). These results were generally similar to the real-time PCR results. The level of AIF mRNA expression in the mesometrial decidua was stronger than in the antimesometrial decidua. When a mouse AIF sense probe was used in place of the antisense probe, there was no detectable signal in the uterus (Figure 2 control).

The immunohistochemistry results showed that AIF protein (green fluorescence) was present in almost all uteri during early pregnancy. A basal level of AIF protein was observed in the LE and GE on day 1 of pregnancy (Figure 3D1). The signal was higher in the LE and GE from days 2 to 4 of pregnancy (Figure 3D2, D3, D4). On day 5 of pregnancy, a moderately strong AIF immunostaining signal was observed in the subluminal stroma immediately

surrounding the implanted blastocyst (Figure 3D5). From

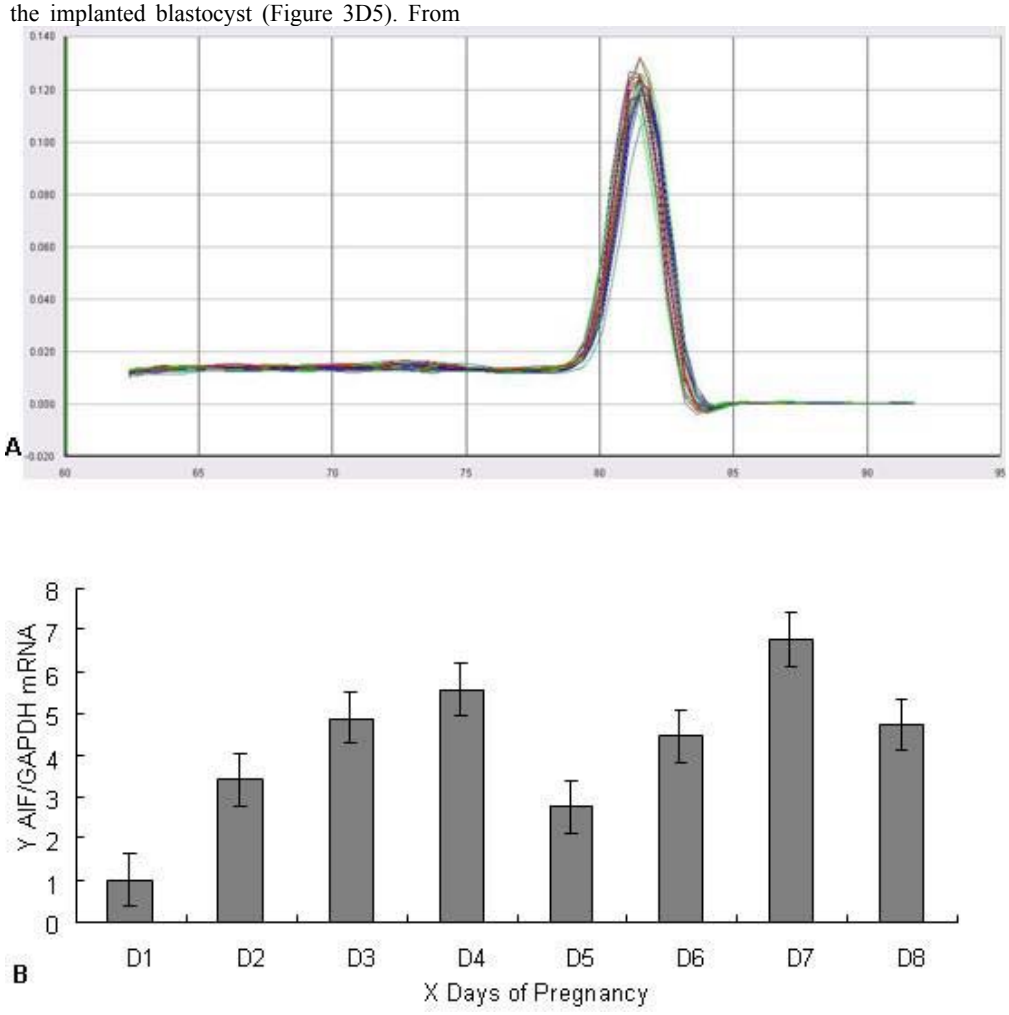

Figure 1. A. Melting curve of real-time quantitative PCR analysis for AIF mRNA. The PCR products exhibited melting curves indicating the amplification of a single product. B. Uterine expression level of AIF mRNA during early pregnancy. Real-time quantitative PCR analysis of the relative expression level of AIF mRNA in mouse uterine tissues on day 1 (D1), day 2 (D2), day 3 (D3), day 4 (D4), day 5 (D5), day 6 (D6), day 7 (D7) and day 8 (D8) of pregnancy, using GAPDH as the housekeeping gene (statistical significance for the expression on each day is versus that on day 1 of pregnancy).

days 6 to 8 of pregnancy, a high level of AIF immunostaining appeared, not only in the PDZ but also in the SDZ and gradually increased (Figure 3D6, D7, D8). There was no detectable AIF protein signal when goat anti-AIF IgG was replaced with normal horse IgG (data not shown). To further determine the subcellular localization of AIF, uterine sections were subjected to immunofluorescent staining with an anti-AIF FITC antibody together with DAPI nuclear staining. The AIF and DAPI stains localized to distinct compartments consistent with the cytoplasm and nucleus, respectively (data not shown).

5.2. AIF expression in the mouse uterus during pseudopregnancy

The *in situ* hybridization results showed that AIF mRNA appeared in the LE and GE from days 1 to 5 of pseudopregnancy (data not shown). Immunohistochemistry results showed that AIF protein signals appeared in almost all uteri. The tendency of the expression pattern was similar to that observed on days 1 to 4 of pregnancy.

5.3. AIF expression in the mouse uterus under delayed implantation and activation

The *in situ* hybridization results showed that there was AIF mRNA expression in the LE and GE under delayed implantation conditions (Figure 4A). After delayed implantation was terminated by estrogen treatment and embryos were implanted, a strong AIF mRNA signal was observed in the subluminal stroma around the implanted blastocyst (Figure 4B).

The immunohistochemistry results showed that AIF protein (green fluorescence) was detected in almost all uteri under both delayed and activated conditions. A moderately strong AIF immunostaining signal was detected in the subluminal stroma immediately surrounding the

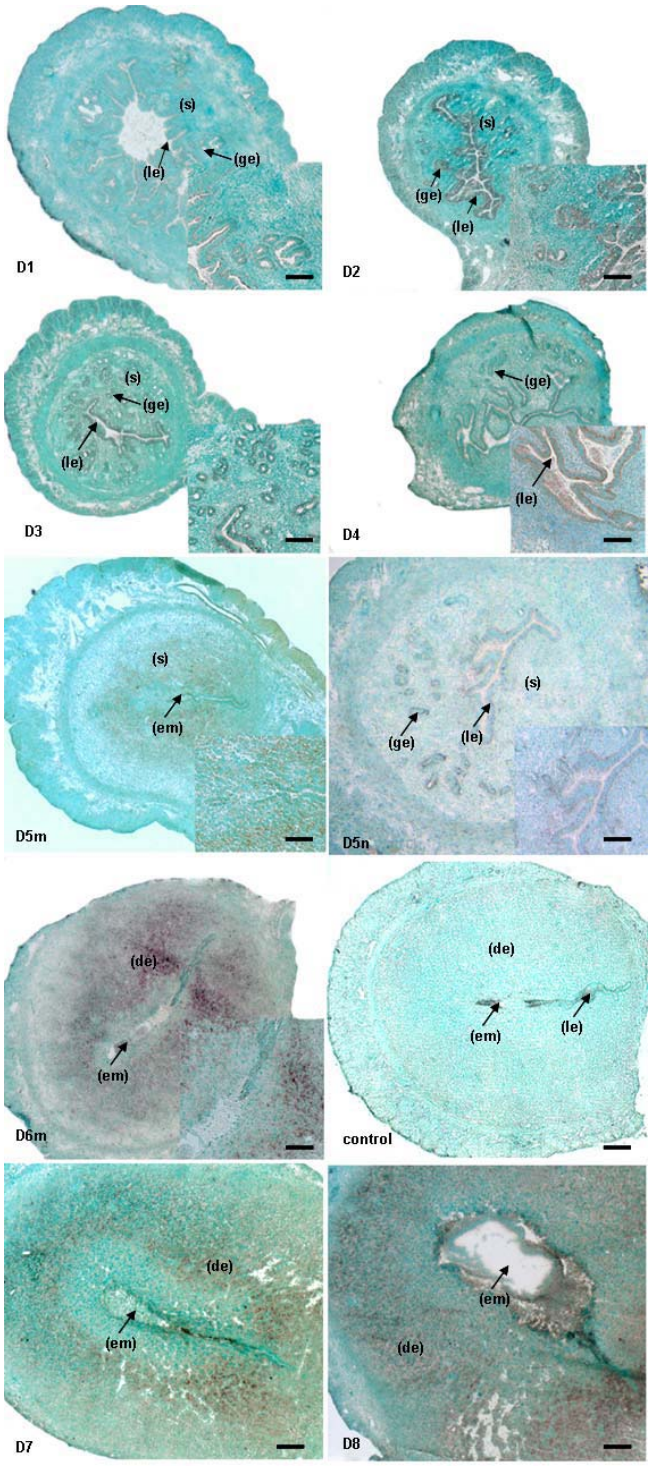

Figure 2. *In situ* hybridization of AIF mRNA in mouse uteri during early pregnancy. There was a basal level of AIF mRNA expression in the LE and GE on day 1 of pregnancy (D1). The signal in the LE and GE from days 2 to 4 of pregnancy became stronger (D2, D3, D4). An AIF mRNA signal was not detected in the LE but was detected in the stromal cells surrounding the embryo (D5m). However, there was a low level of AIF mRNA expression in the LE at inter-implantation sites (D5n). From days 6 to 8 of pregnancy, the signal was strongly confined to the SDZ and gradually increased (D6m, D7, D8); control is the same as D6m, but a sense sequence was used as a control. le, luminal epithelium; ge, glandular epithelium; s, stroma ; de, decidua; em, embryo. Bar=200μm.

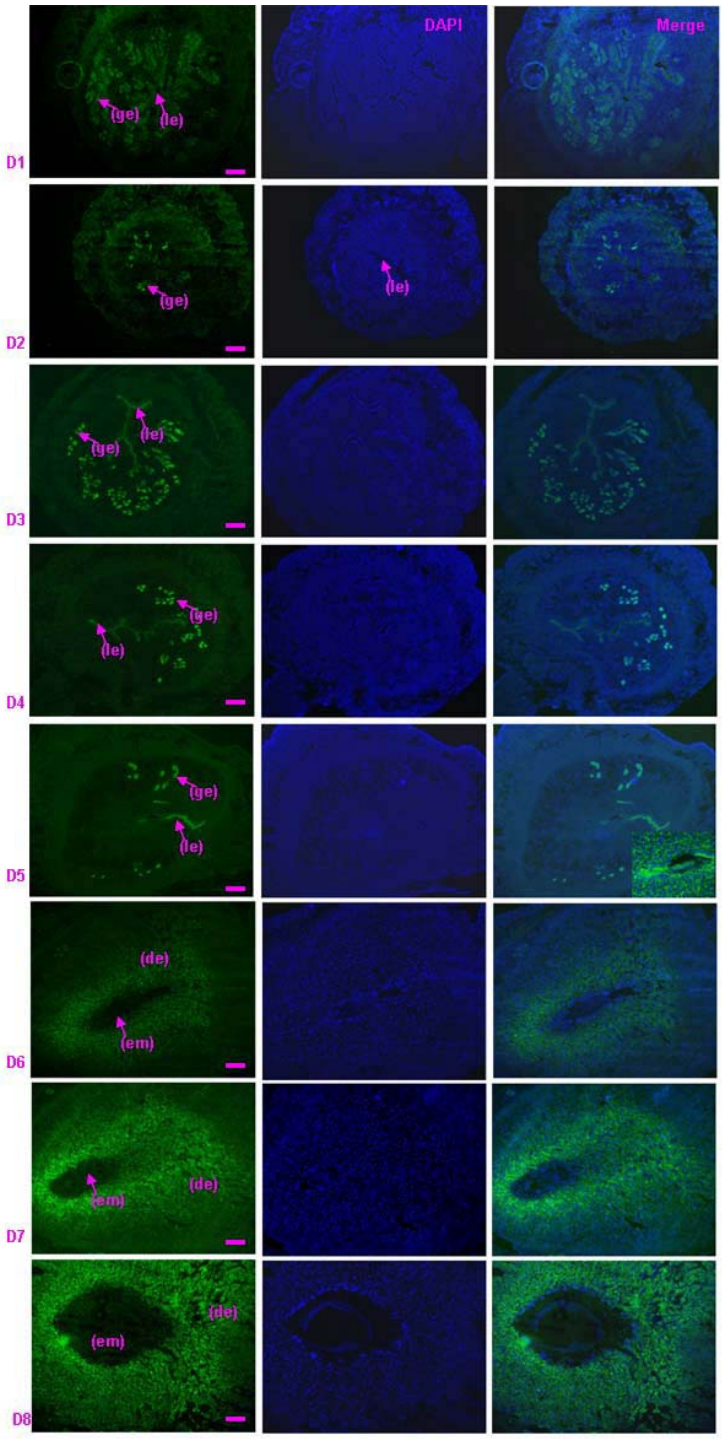

Figure 3. Immunohistochemistry of AIF in the mouse uterus during early pregnancy. AIF protein (green fluorescence) was visualized in almost all uteri. A basal level of AIF protein was observed in the LE and GE on day 1 of pregnancy (D1). The expression of AIF protein became stronger in the LE and GE from days 2 to 4 of pregnancy (D2, D3, D4). A moderately strong AIF immunostaining signal was observed in the subluminal stroma immediately surrounding the implanted blastocyst (D5). From days 6 to 8 of pregnancy, a high level of AIF immunostaining appeared, not only in the PDZ but also in the SDZ and gradually increased (D6, D7, D8, respectively). Goat anti-AIF IgG was replaced by normal horse IgG as a control (data not shown). The AIF proteins signals were visualized as green fluorescence, and the nucleus was visualized based on blue fluorescence under a microscope. le, luminal epithelium; ge, glandular epithelium; s, stroma.Merged images are also shown in the third row. Bar=100μm.

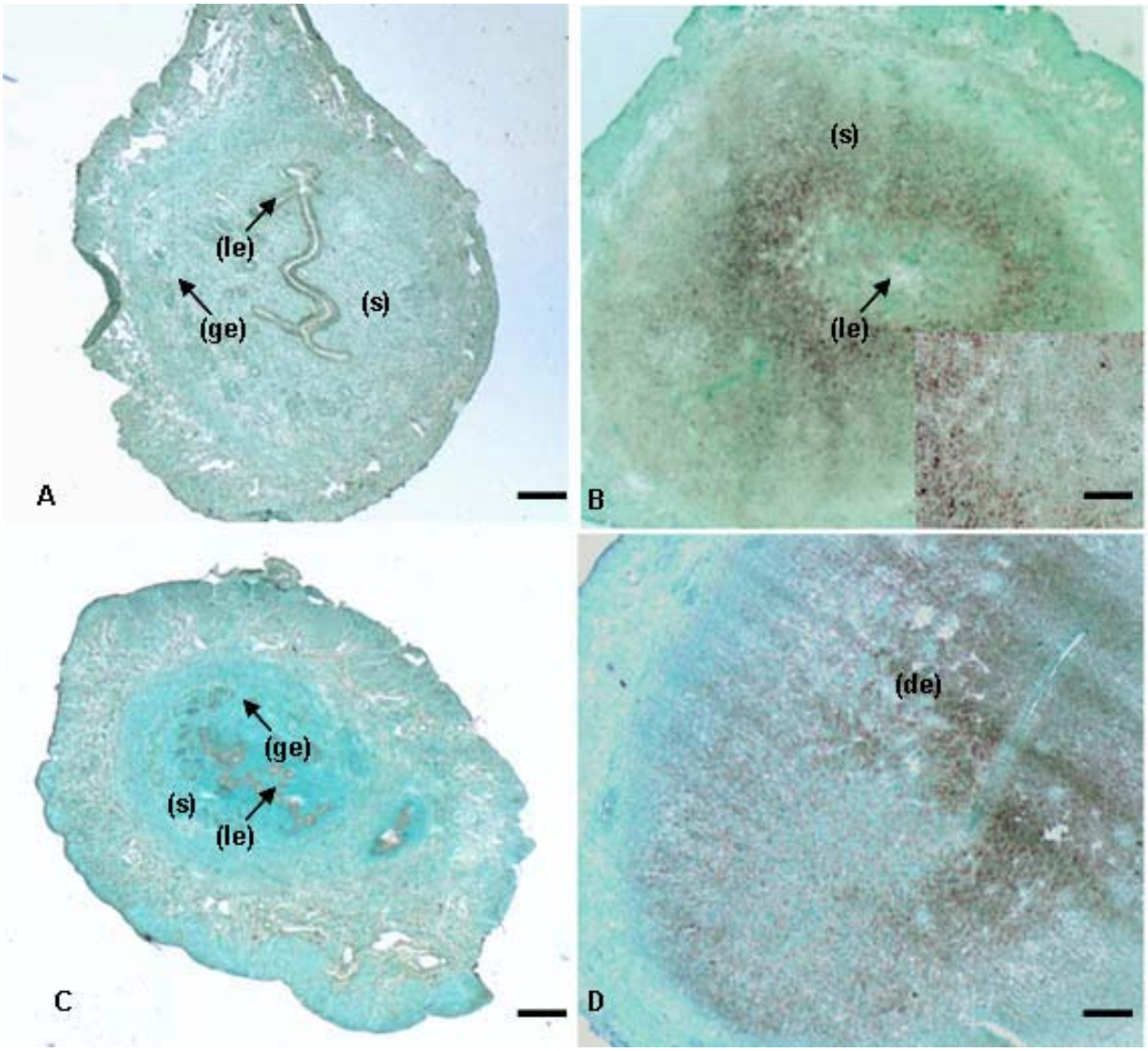

Figure 4. *In situ* hybridization of AIF mRNA in the mouse uterus under delayed, activated implantation and artificial decidualization. AIF mRNA staining was detected in the LE and GE under delayed implantation conditions (A). After delayed implantation was terminated by estrogen treatment and embryos implanted, a strong AIF mRNA signal was observed in the subluminal stroma around the implanted blastocyst (B). An AIF mRNA signal was strongly detected in the decidual zone under artificial decidualization, and its expression in the mesometrial decidua was stronger than in the antimesometrial decidua (D). AIF mRNA staining appeared in the LE and GE but not in the stroma, of control uterine samples (C). le, luminal epithelium; ge, glandular epithelium; s, stroma. Bar=200μm.

implanted blastocyst (data not shown), and the expression pattern was similar to that observed on day 5 of pregnancy.

5.4. AIF expression in the mouse uterus under artificial decidualization

The *in situ* hybridization results showed that AIF mRNA appeared in the LE and GE, but not in the stroma, of control uterine samples (Figure 4C). A strong AIF mRNA signal was detected in the decidual zone under artificial decidualization, and its expression in the mesometrial decidua was stronger than in the antimesometrial decidua (Figure 4D). There was also strong AIF immunostaining signal in the decidual zone (Figure 5A). In merged pictures, the AIF and DAPI stains were localized to the cytoplasm and nucleus, respectively (Figure 5B, D3).

5.5. Effects of steroid hormones on AIF expression

The *in situ* hybridization and immunohistochemistry results showed that the expression of AIF mRNA and protein gradually decreased in the LE and GE of the mouse uteri under E2 treatment after 2 h, 6 h, 12 h and 24 h (Figure 6A, B, C, D and Figure 7A, B, C, D). Compared with E2 treatment, P4 treatment resulted in a

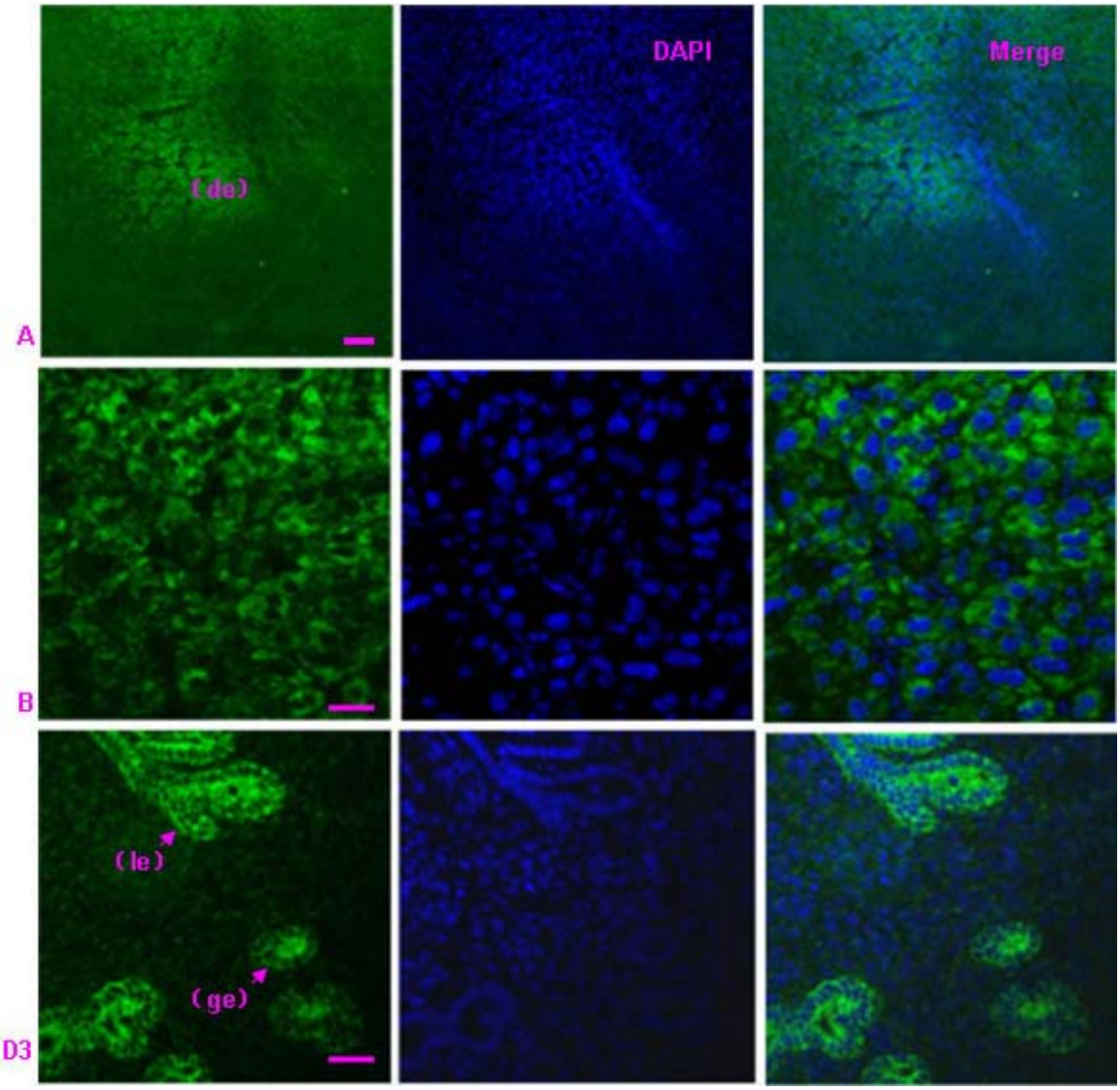

Figure 5. Immunohistochemistry of AIF protein in the mouse uterus under artificial decidualization and on day 3 of pregnancy. There was strong AIF immunostaining in the decidual zone (A). AIF protein signals were all found to be in the cytoplasm and not in the nucleus of the stroma (B), LE or GE (D3) cells. le, luminal epithelium; ge, glandular epithelium. Merged images are also shown in the third row. Bar=100μm.

sustained stable level of AIF mRNA (Figure 6E, F, G, H) and protein (data not shown) expression in the LE and GE. Under a combination of E2 and P4 stimulation, the AIF mRNA and protein levels in the LE and GE of the mouse uteri (Figure 6N and Figure 7F) were similar to those observed in oil-treated uteri (Figure 6M and Figure 7E). AIF protein (green fluorescence) was located in the cytoplasm.

6. DISCUSSION

The immunohistochemistry and *in situ* hybridization results showed that the expression of AIF was temporally and spatially regulated in the mouse uterus on days 1 to 8 of pregnancy. This result implies that AIF might take part in the process of embryo implantation. On days 5 to 8 of pregnancy, AIF mRNA and protein were strongly detected in the uterine stroma. On days 6 to 8 of pregnancy, there was a strong signal for AIF mRNA expression that was mainly located in the SDZ. As pregnancy progressed and the number of decidual cells increased, the area including AIF-positive cells increased. This suggests that AIF might be associated with decidualization. It is well known that the decidual process is characterized by the spatially coordinated progression of the proliferation and differentiation of endometrial fibroblast-like stromal cells into decidual cells (17), and the SDZ represents an extension of the decidualization process where stromal cell proliferation and differentiation continue. This implies that AIF might not exert its pro-apoptotic function during this process.

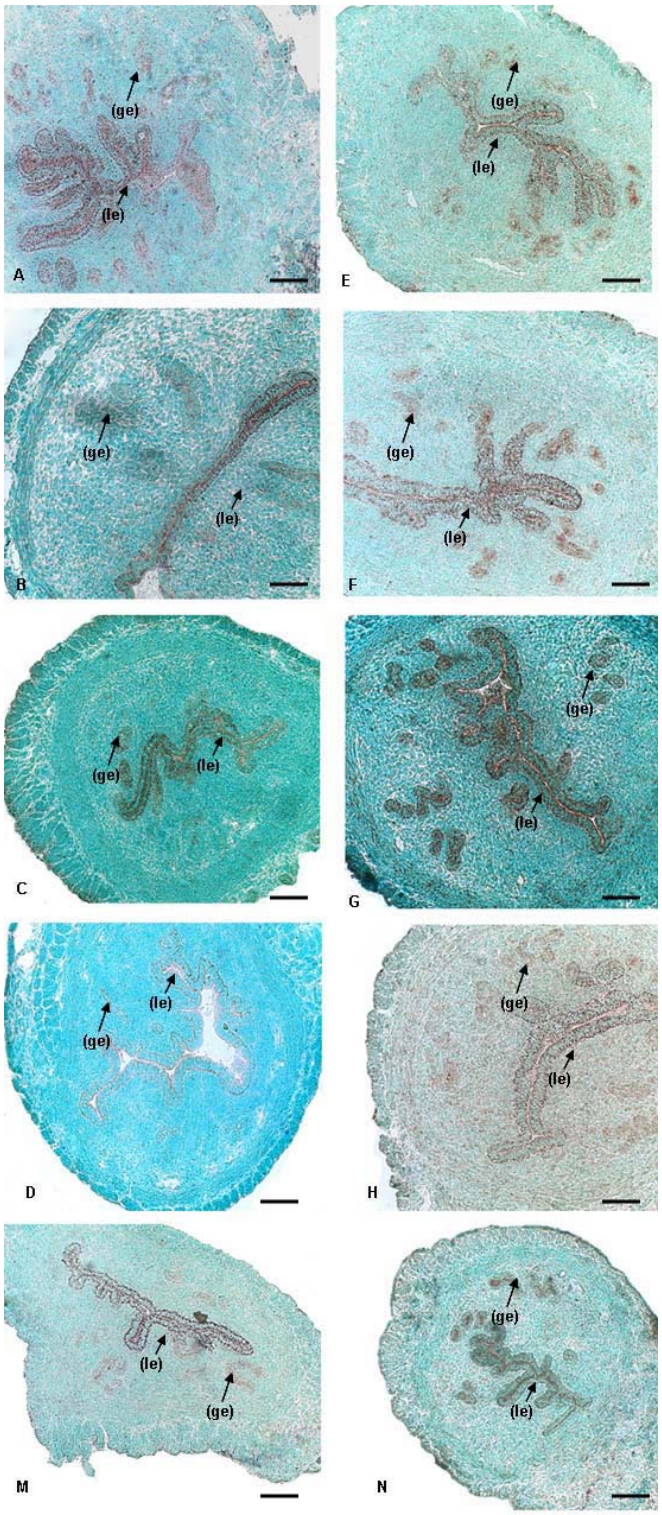

Figure 6. *In situ* hybridization of AIF mRNA in the mouse uterus under steroid hormone treatment conditions. AIF mRNA was observed in the LE and GE. AIF mRNA staining gradually decreased in mouse uteri under E2 treatment after 2 h, 6 h, 12 h and 24 h (A, B, C, and D, respectively). AIF mRNA expression was sustained at a stable level in the LE and GE of mouse uteri under P4 treatment (E, F, G, H, respectively). AIF staining under a combination of E2 and P4 stimulation (N) appeared similar to that observed in oil-treated uteri (M). le, luminal epithelium; ge, glandular epithelium. Bar=100μm.

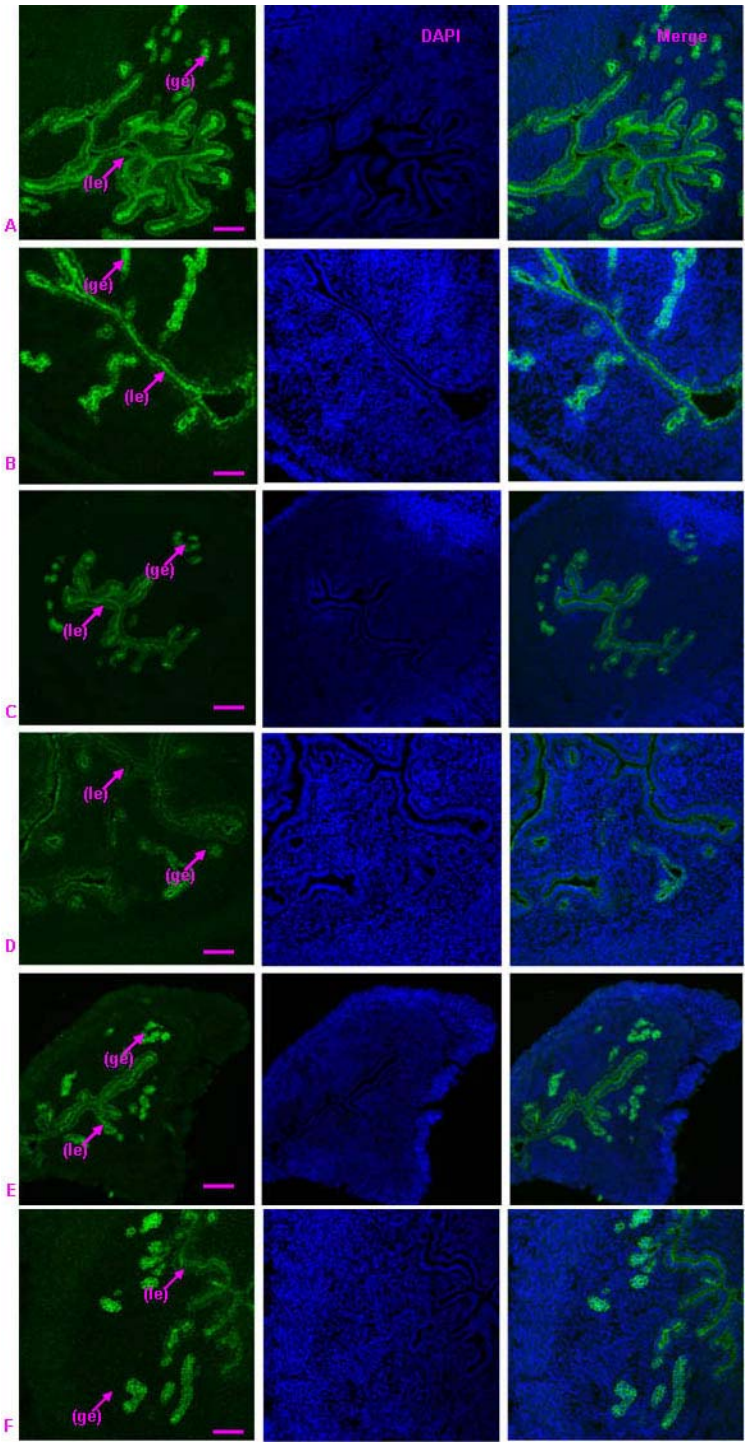

Figure 7. Immunohistochemistry of AIF protein in the mouse uteri under E2 treatment. Mouse uteri were treated with E2 for 2 h (A), 6 h (B), 12 h (C) or 24 h (D). AIF protein was observed to gradually decrease in the LE and GE (A, B, C, and D, respectively). A control treated for 24 h with oil, which exhibited a moderate level of AIF protein in the LE and GE (E). A combination of E2 and P4 stimulation (F) resulted in a similar expression pattern as that induced by oil treatment. Additionally, only a low level of AIF immunostaining was detected in the stroma. The AIF proteins signals were visualized as green fluorescence, and the nucleus was visualized based on blue fluorescence under a microscope. le, luminal epithelium; ge, glandular epithelium. Merged images are also shown in the third row. Bar=100μm.

Embryo implantation is regulated not only by cell proliferation and differentiation but also by programmed cell death, or apoptosis. Classically, apoptotic pathways have been described as either intrinsic or extrinsic. In the intrinsic pathway, the death signal is generated from within the cell, such as in the case of cell death caused by genotoxic stress (18). Mitochondrial membrane permeabilization (MMP) is an essential key feature of the intrinsic pathway. Following MMP, genotoxic stress in the nucleus can induce AIF to be released from the mitochondrion to the nucleus and mediate apoptosis-like PCD (7, 19). AIF is a protein that regulates caspase-independent apoptosis (20). In contrast, in the extrinsic pathway the death signal is generated by the binding of a death ligand to a cell surface receptor, which then activates the apoptotic cascade (21). In cases where death ligands bind to surface receptors that then activate caspase, cell death can proceed independently of the mitochondrion and without MMP (21).This course does not rely on the release of AIF. Embryo implantation is a mutual interaction between the blastocyst and the uterus, and embryo-derived cues may trigger uterine events. Joswig *et al.* found that the apoptotic events involved in uterine epithelial and in decidual programmed cell death seem to be mediated by TNF receptor 1 followed by caspase-3, Fas ligands and caspase-9 (22). Moreover, ligand-receptor signaling through TGF-beta can induce decidual cell apoptosis through caspases (23). The apoptotic degeneration of the uterine epithelium and stromal cells during pregnancy seems to be caused by the extrinsic pathway, which can proceed without the release of AIF. AIF might not play its apoptotic function in the uterus during this process.

Our immunohistochemistry results showed AIF protein signals in almost all uteri. The antibody (anti-AIF, sc9416) we used can detect 67 kDa, 62 kDa and 57 kDa versions of AIF protein (15). During implantation, AIF protein signals were observed in the cytoplasm and not in the nuclei of uterine cells, which indicates that AIF was not transported to the nucleus in these cells. As AIF transport into the nucleus is necessary for its pro-apoptotic activity, AIF would be unable to promote apoptosis in these cells. Because it is localized to the cytoplasm during early pregnancy, AIF might act in its pro-survival capacity.

Based on the AIF expression pattern observed in the SDZ in our *in situ* hybridization experiments, the subcellular localization of AIF protein in the cytoplasm instead of the nucleus found by immunohistochemistry and the nature of the apoptotic pathways, we speculated that, rather than helping to induce apoptosis in uterine cells during mouse embryo implantation, AIF may instead be acting in its physiological capacity.

The uterine environment exhibits a lower O_2 concentration than the oviduct. Embryos encounter a decreasing O_2 concentration gradient as they progress along the reproductive tract. During early implantation, hypoxic and even anoxic conditions confront the invading trophectoderm (24). The uterus is known to possess peroxidase activity in the endometrium, which involves oxygen consumption and an increase in NAD(P)H oxidation-producing superoxide anions (O_2^-), which are then dismutated to H_2O_2 (25). Ishikawa and his colleagues suggested that there were uterine enzymes, most likely of the nature of uterine H_2O_2-generating NAD(P)H oxidase. Coincidently, AIF exhibits ROS-generating NADH oxidase activity and reacts rapidly with oxygen, forming O_2- (4). Our results showed that an AIF signal appeared in the LE on days 1 to 4 of pregnancy, which suggests that AIF could have some connection with the O_2 concentration in the mouse uterus. Whether AIF exhibits NADH oxidase activity and is associated with uterine oxygen consumption and H_2O_2 generation in the mouse uterus need to be further studied.

An AIF mRNA signal was detected in the subluminal stroma immediately surrounding the implanting blastocyst on day 5 of pregnancy, whereas it was not detected in the uterine stroma at interimplantation sites, on day 5 of pseudopregnancy or under delayed implantation. After delayed implantation was terminated by estrogen treatment and blastocysts became implanted, AIF mRNA appeared in the subluminal stroma surrounding the implanting blastocyst, similar to what was observed on day 5 of pregnancy. Our data suggest that the AIF mRNA expressed in the stroma was regulated by the active blastocysts during the peri-implantation period.

Our *in situ* hybridization results showed that an AIF mRNA signal appeared in the SDZ during pregnancy but not in the PDZ. However, under artificial decidualization the signal was detected in the entire decidual zone, not only in the SDZ but also in the PDZ. The endometrial tissue associated with artificially induced decidualization is referred to as a deciduoma. Several studies have highlighted differences between decidua and deciduoma formation in rodents, such as small morphohistological differences and gene expression differences in the deciduoma (26). The AIF mRNA expression pattern observed herein also indicates that there are some differences between decidualization and deciduomas. Furthermore, the artificial decidualization model should be improved to produce the best deciduoma model in which the morphohistological and molecular changes are spatially and temporally similar to those observed during formation of the decidua in the endometrium.

In our hormonal treatment models, the expression of AIF in the LE and GE was downregulated by E2 in a time-dependent manner, and a stable level was sustained under P4 treatment alone. A combination of E2 and P4 stimulated a similar expression pattern as was observed in controls. This implied that AIF expression in the LE and GE in the mouse uterus might be regulated by steroid hormones. Implantation has long been known to be a steroid hormone-dependent process. The coordinated activity of both estrogen and progesterone is necessary both before and during the process of implantation in the mouse endometrium. On days 1 to 4 of early pregnancy, the expression of AIF observed in the LE and GE may be due to the combined action of estradiol secreted from the ovary and progesterone secreted from the corpus luteum. On day

5 of pregnancy, the finding of no AIF mRNA signal in the LE might be the result of preimplantation ovarian estrogen secretion on day 4 of pregnancy.

Molecules expressed in the uterus could regulate the function of AIF. Akt and its phosphorylated form have been found to be present in human endometrial stromal cells, which may play a role in decidualization. Moreover, Hsp70 also participates in the proliferation of stroma cells in endometrial decidualization and has been found to be closely tied to decidualization. The translocation of AIF from the mitochondria to the nucleus is inhibited by Akt during CDDP or ceramide-induced apoptosis in chemosensitive cells (8). Cytoplasmic Hsp70 specifically interacts with exogenous and endogenous AIF and inhibits its relocation to the nuclear compartment (reviewed in (3)). Thus, the process of AIF translation to the nucleus could also be inhibited by Akt or Hsp70 in mouse uterine decidualization cells.

In conclusion, AIF could play an important role during the peri-implantation period and might be associated with decidualization. During early pregnancy, AIF may be exerting its physiological activity rather than carrying out its pro-apoptotic function in the mouse uterus. AIF expression in the LE and GE is influenced by steroid hormones. AIF mRNA expression in the stroma is regulated by the active blastocyst. There are some differences in the expression pattern of AIF under decidualization versus artificial decidualization.

7. ACKNOWLEDGEMENTS

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Abbreviations: AIF: apoptosis-inducing factor; PDZ: primary decidual zone; SDZ: secondary decidual zone; LE: luminal epithelium; GE: glandular epithelium; MMP: mitochondrial membrane permeabilization; TNF: tumor necrosis factor; TGF-beta: transforming growth factor beta; Hsp70: heat shock protein 70; P4: progesterone; E2: 17-beta estradiol

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