Enhanced Polyadenosine Diphosphate-Ribosylation in Gonadotropin-Releasing Hormone Agonist-Treated Uterine Leiomyoma

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This study aimed to examine the activation of poly(ADP-ribose) polymerase (PARP) and the accumulation of its end product, poly(ADP-ribose) (PAR), in uterine leiomyoma specimens obtained from 25 patients receiving Leuprolin depot (leupreolin acetate, depot (LA)) treatment and 46 control patients and explore their correlation with tumor shrinkage and degeneration caused by the therapy. Immunoblotting analysis showed that specimens from LA-treated patients had higher PARP expression. The numbers of both PARP- and PAR-immunolabeled cells were higher in leiomyoma with LA treatment. This was correlated with the clinical response of LA therapy that LA induced more leiomyoma degeneration. The analysis of power Doppler sonography indicated a progressive decrease in blood supply to tumor following LA treatment. In vitro experiments using primarily cultured leiomyoma cells exhibited that the deprivation of serum or ovarian hormones or LA directly failed to induce PARP and PAR production. Our results suggested that reduced blood flow and subsequent ischemic damages in leiomyoma could be responsible for PARP overexpression and PAR accumulation, clinical response, and tumor degeneration caused by LA treatment. (J Clin Endocrinol Metab 88: 5009–5016, 2003)

Uterine leiomyoma, a common pelvic tumor of reproductive age that sometimes causes significant morbidity, is a smooth muscle tumor with increased cellularity and hypertrophy of the cells (1). Its growth depends on ovarian steroid hormones and growth factors. Studies have demonstrated that monthly administration of a long-acting GnRH agonist (GnRH-a) on patients with uterine leiomyomas reduces tumor sizes and the levels of ovarian hormones and some growth factors that are required for tumor growth (2–4).

Although the molecular mechanism by which GnRH-a acts on uterine leiomyomas has not been fully resolved, it is suggested that the effect of GnRH-a on reducing tumor volume can be mediated by the induction of cellular atrophy of leiomyoma cells (5) and/or the decrease of the tumor cell number (probably resulting from apoptotic/necrotic cell death and mitosis suppression) (6, 7). Our group and others (8–10) have demonstrated that GnRH-a treatment of the uterine leiomyoma did not increase apoptotic cell death involving the interaction of Fas and its ligand, caspases, and Bcl2. In addition, accumulating evidence points to subsequent cell damage (5), degeneration, and necrosis (7) that contribute to tumor shrinkage in the aftermath of GnRH-a therapy.

One reported event following GnRH-a therapy is the reduction of blood flow to the uterus (11, 12). It is because a hypoestrogenic environment caused by GnRH-a has increased vascular resistance, leading to the subsequent decrease of blood supply to leiomyoma nodules (12, 13). Although ischemia causes oxidative stress that in turn damages DNA, DNA repair and other protective mechanisms in the cells would be turned on to avoid the replication of impaired DNA (14). For most of the cells, such damage can be just sublethal and reversible. For injured cells with energy supply depletion, however, mortality ensues reasonably.

Poly(ADP-ribose) (PAR) polymerase (PARP), a nuclear enzyme relating to DNA repair, is activated by and binds single- or double-strand DNA breaks (15). Experimental studies indicate that PARP overactivation in response to oxidative damage of DNA can stimulate DNA repair, silence gene transcription for cell growth when ADP-ribose polymers accumulate, and enhance ATP generation from NAD+ to maintain cell survival (16, 17).

Because a PARP requires one molecule of NAD+ to transfer each ADP-ribose unit and NAD+ regeneration demands four molecules of ATP, thereafter prolonged PARP activation in injured cells therefore causes a massive consumption of NAD+ and ATP and leads to cell death by necrosis as a result of energy depletion (18–21).

In this article, we investigated the expression profiles of PARP and PAR in leiomyoma cells as well as control cells with or without GnRH-a therapy and then explored how these expression profiles correlated with tumor responses to the therapy and some pathological findings relating to cell injury such as degeneration and necrosis. The changes of blood supply to the leiomyoma were assessed by three-dimensional power Doppler ultrasonography. In addition, in
vitro primary cultures of leiomyoma were exploited to study the effect of ovarian hormones, LA, or hypoxic insults on the expression of PARP and PAR.

Patients and Methods

Tissue collection, characterization, and tissue cell lysate preparation

From January 1998 to December 2001, we prospectively collected patients who were treated with three doses of Leuplin Depot [leuprolin acetate, depot (LA), Takeda Chemical Industries, Ltd., Tokyo, Japan], a long-acting GnRH-a, for approximately 2–3 wk before myomectomy at the National Cheng Kung University Hospital (Tainan, Taiwan) as the LA-treated group. Women with regular menstrual cycle undergoing hysterec- tomy or myomectomy during the follicular phase of the cycle without previous exogenous hormonal or GnRH-a therapy were selected as the control group. A specimen pair of leiomyoma and myometrium belonging to each patient of either the LA-treated group or the control group was obtained from the surgeries in the respective conditions mentioned above. The works of these investigations were conducted after obtaining informed consent from patients and approval from the institutional review board at National Cheng Kung University Hospital. The representative tissue samples were taken from distinct nondegenerating leiomyomas and a separate site 1 cm away from the tumor as normal myometrium. The final diagnosis of uterine leiomyomas was confirmed by the pathologic findings in each case. The specimens of tissue lysate from frozen tissues were prepared for further analysis as described previously (10).

Ultrasonographic examination

All of the patients of both groups underwent ultrasonographic examination using an end-viewing 90 degree-angled real-time convex 5-MHz transvaginal transducer or a 3.5-MHz transabdominal probe as an adjunct to the transvaginal probe (Aloka SSD-680, Aloka, Tokyo, Japan), a long-acting GnRH-a, for approximately 2–3 wk before myomectomy at the National Cheng Kung University Hospital (Tainan, Taiwan) as the LA-treated group. Women with regular menstrual cycle undergoing hysterec- tomy or myomectomy during the follicular phase of the cycle without previous exogenous hormonal or GnRH-a therapy were selected as the control group. A specimen pair of leiomyoma and myometrium belonging to each patient of either the LA-treated group or the control group was obtained from the surgeries in the respective conditions mentioned above. The works of these investigations were conducted after obtaining informed consent from patients and approval from the institutional review board at National Cheng Kung University Hospital. The representative tissue samples were taken from distinct nondegenerating leiomyomas and a separate site 1 cm away from the tumor as normal myometrium. The final diagnosis of uterine leiomyomas was confirmed by the pathologic findings in each case. The specimens of tissue lysate from frozen tissues were prepared for further analysis as described previously (10).

Immunoblotting analysis

Because the tissue lysate might contain a variety of proportion of extracellular protein, intracellular β-actin was used to ensure the even loading of protein samples on SDS-polyacrylamide gel. The protein samples separated by SDS-PAGE were then transferred onto nitrocel- lulose membranes by a semidry blotting system. The membranes were blocked in 1 × TBS containing 5% (wt/vol) skim milk at room temperature for 1 h, washed in a mixture of PBS and 0.05% Tween 20 (Twen- PBS; Sigma Chemical, St. Louis, MO) and then incubated overnight at room temperature with PARP antibody (Transduction Laboratories, Lexington, KY) for 1 h. After being washed in Tween-PBS, the mem- branes were incubated with 1000-fold diluted, biotinylated antimouse IgG antibody conjugated with horseradish peroxidase (Amersham Pharm- macia Biotech Inc., Piscataway, NJ) at room temperature for 1 h and then developed by the enhanced chemiluminescence system (Amersham Pharmacia Biotech Inc.). The intensity of each band in Western blot was then analyzed by a laser densitometer (PD-120, Molecular Dynamics, Sunnyvale, CA). To obtain comparable data from different films, relative intensity was used and defined as the ratio of band intensity of each lane to that of its corresponding β-actin.

PARP and PAR immunostaining of surgical specimens

After surgery the surgical specimens were sent for histological examination as soon as possible. To reduce the heterogeneity of clinical specimens, sections of 3 μm in thickness were cut from each formalin-fixed, paraffin-embedded tissue block that contained the largest tumor nodule of each patient. For each tumor, one section was taken and stained with hematoxylin-eosin for histological confirmation of tumor presence and evaluation of histological changes after LA treatment. The adjacent sections were stained with PARP or PAR antibody according to the standard immunoperoxidase staining methods. The presence of leiomyomas and histologic findings such as hyaline and hydropic degeneration on the slides were reviewed by one investigator (C.-L.H.), who was blind to patient status of each sample. Hyaline degeneration was defined as a nodular confluent acellular area, and hydropic degener- ation is a geographically distributed edematous zone that probably preceded hyaline degeneration (7). The pathologic changes were graded according to the percentage of degeneration area in a ×100 field and des- ignated as follows: grade 1, 0–5%; grade 2, 5–20%; grade 3, more than 20%.

Monoclonal antibodies against PARP (Transduction) and PAR (BIOMOL Research Laboratories, Inc., Plymouth Meeting, PA) were used to stain the histological slides from the paraffin blocks as described elsewhere with minor modifications (23). Briefly, sections were depara- finized and treated with methyl alcohol and hydrogen peroxide to block endogenous peroxidase activity. Section-bearing slides placed in 0.1 mmol/liter, pH 6.0 citrate buffer (Antigen Retrieval Citra, Bio- Genex, San Ramon, CA) were heated in a microwave oven (Bio-Rad 1250 microwave, Energy Beam Science, Richmond, CA) at 97% maximal power level, at 97 C for two 5-min cycles with an interval of

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Primary culture of leiomyoma cells

Primary explant cultures of leiomyoma were isolated from leiomyoma tissue and maintained as described previously (22). In brief, the leiomyoma tissue was minced into 1- to 2-mm3 explants, placed in 6-cm petri dishes and cultured with DMEM (Life Technologies, Grand Island, NY) plus 20% fetal bovine serum (FBS). Dishes were left undisturbed for 1 wk and then examined for the evidence of cell outgrowth. After cell confluence, cells were transferred to culture flasks containing DMEM plus 10% FBS. These smooth muscle-like cells were confirmed to have the characteristic features of uterine muscle cells as follows: fusiform shape, expression of smooth muscle-specific α-actin, and estrogen re- sponsiveness. All the cultured cells harvested for in vitro experiments were either nonpassaged cells or those in two to four subcultures. The growth medium in the culture dishes with leiomyoma cells was replaced with serum-free and phenol red-free DMEM 24 h before experiments.

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tumor reduction was 76 ml, and the volume reduction rate of individual tumor nodule varied from 8–97%. Five patients had a tumor volume reduction less than 20% and were deemed as poor responders. The mean volume of leiomyomas in LA-naïve patients was 150.3 ± 71.8 ml (ranging from 14–323 ml). There was no significant difference between the two groups on the aspect of age (P > 0.05, unpaired t test) and tumor size (P > 0.05, unpaired t test) before LA treatment.

Expression of PARP and PAR and their correlations with degeneration of leiomyomas

Figure 1 shows the representative results of four pairs of myometria and leiomyomas from two LA-treated patients and two LA-untreated patients in Western blot analysis for PARP, in which significant bands referring PARP are noted in relative intensities. The statistical data of all these 25 LA-treated patients and 46 LA-untreated patients are also summarized in Fig. 1. The LA-untreated leiomyomas had an up-regulated expression of PARP as compared with their homologous myometria (P < 0.05, paired t test). The expression levels of PARP in the LA-treated leiomyomas were observed significantly higher than those in the nontreated controls (P < 0.001, unpaired t test).

Immunohistochemical staining of PARP (Fig. 2A) and PAR (Fig. 2C) found that almost all of the stain was confined in cell nucleus of leiomyoma. Compared with myometria, leiomyomas were more frequently detected as PARP-positive cells in particular around the degenerative areas of the

Results

Clinical findings

During the study period, a total of 25 consecutive patients receiving preoperative LA treatment were collected. The patient number of control group (without preoperative LA treatment) collected in the same study session was 46. The patients of both groups were normally distributed in tumor volumes. The age of patients was 29–37 yr (mean = 33) in the LA-treated group and 32–43 yr (mean = 37) in the control group. The mean tumor volume before LA treatment was 187.4 ± 94.2 ml (ranging from 33–413 ml), whereas the mean volume after therapy was 88.4 ± 50.8 ml (ranging from 16–203 ml). LA significantly reduced the leiomyoma tumor volume (P < 0.001, paired t test). The median volume of
tumors. Besides, PAR-staining positive cells far exceeded PARP-staining positive cells in number.

Among the 25 LA-treated patients, the PARP-staining grades and the patient numbers of particular grades are described as follows: grade 3 (seven patients), grade 2 (11 patients), and grade 1 (seven patients); and the PAR-staining grades and their patient numbers: grade 2 (16 patients) and grade 1 (nine patients). It was seemly that the results of PARP staining were parallel with those of PAR staining. Among the 46 LA-untreated patients, the PARP-staining grades and the corresponding patient numbers were: grade 3 (three patients), grade 2 (13 patients), and grade 1 (30 patients); and the PAR-staining grades and their patient numbers: grade 2 (12 patients) and grade 1 (34 patients). Statistical analyses revealed that leiomyomas with LA treatment expressed more PARP and PAR as compared with nontreated controls (P < 0.001 and P < 0.001, respectively; Mann-Whitney U test). In addition, the extent of tumor shrinkage was positively cor-

3-D power Doppler sonography

We used 3-D color Doppler sonography examination to measure the change of blood flow to tumor before and during LA treatment. We found that more than 90% of leiomyomas in the 16 LA-treated patients had a peripheral distribution of vascular supply and that a progressive decrease of the blood flow to tumor could be observed in the serial follow-up examination. Figure 4 shows the serial changes of blood flow patterns, tumor angiography, and blood-flowing parameters in tumor detected by 3-D power Doppler sonography in a representative patient receiving LA treatment. The preliminary analysis on the changes of blood flow parameters in uterine leiomyoma revealed that average VI decreased from 1.14 ± 0.76 to 0.46 ± 0.31 (P < 0.001; Wilcoxon signed ranks test), FI decreased from 50.77 ± 5.00 to 38.48 ± 8.98 (P < 0.001; Wilcoxon signed ranks test), and VFI decreased from 0.59 ± 0.39 to 0.23 ± 0.17 (P < 0.001; Wilcoxon signed ranks test) after three doses of LA treatment.

Effects of sexual steroids, LA, and cobalt chloride on expression of PARP and PAR

Regarding the factors that might be involved in PARP up-regulation and PAR accumulation in LA-treated leiomyomas, the reduction of both blood flow and sex steroid hor-
mones could have played some roles. We used primary cultures of leiomyoma to investigate the effects of serum, LA, estrogen, or cobalt chloride (CoCl2) known as a hypoxia-mimicking agent on the expressions of PAR and PARP. It had been suggested that a substitution of ferrous ions in heme by cobalt ions would cause a conformational change in a heme O2 sensor and induce hypoxic effects (25). When leiomyoma cells were cultured in medium with 10% FBS supplement, PARP and PAR expression were relatively low as shown in the results of Western blot (Fig. 5A) and immunostaining (Fig. 5B). This was in direct contrast to the positive control that exposed to 1 mM H2O2 and exhibited an elevated PARP level in Western blot analysis and more intense stain in the augmented nuclei in the immunostaining results of PARP and PAR. When the growth medium was replaced with serum-free, phenol red-free medium for 5 d, the PARP and PAR expression of leiomyoma cells remained unchanged. Furthermore, cells serum-starved for 5 d were treated with 250 nM 17β-estradiol or 1 nM LA and incubated for another 24 h. The PARP levels in leiomyoma cells were not affected. These indicated that PARP and PAR expressions were dependent on neither GH nor estrogen supplement and LA had no direct effects on their expressions. Of note, the treatment of 200 µM CoCl2 did not induce any elevated PARP levels in the serum-starved leiomyoma cells as long as 5 d. This reflected the fact that hypoxia induced by CoCl2 alone in this study condition was not sufficient to activate PARP, which as widely known could be activated by broken nuclear DNA (26).

Geographical distribution of PARP and PAR in LA-nontreated leiomyomas

The observation that hormonal depletion failed to induce PARP activation led us to suspect that PARP overexpression after LA treatment is more likely to be related to a diminished vascular supply than hormonal depletion. Therefore, we hypothesized that leiomyoma cells in the central part of tumor might be more vulnerable to ischemic damage and should have higher PARP expression than those in the peripheral region because leiomyomas have a peripheral distribution of blood supply. We had assessed the PARP expression of both protein...
peripheral and central parts for 10 specimen pairs of leiomyoma and myometrium of patients whose leiomyoma nodules were larger than 5 cm in diameter. Representative results shown in Fig. 6 indicated that leiomyoma cells at the central part of the tumor had the highest extent of PARP expression, and myometrium had the least PARP expression. These results provide further support for our supposition that PARP expression in leiomyomas is related to tissue ischemia.

Discussion

The growth of uterine leiomyoma depends on growth-supporting hormones and adequate blood supply. GnRH-a is able to bring volume shrinkage to this tumor, although the molecular mechanisms by which GnRH-a acts on uterine smooth muscle cells requires further experimental elucidation. Accumulating evidence has demonstrated that the cellular and pathological changes underlying the mechanism of tumor shrinkage can be mediated by the induction of cellular damage and atrophy (5), the decrease of tumor cell number (6, 7), and the subsequent development of tumor degeneration or necrosis (7). Our previous work has pointed out that GnRH-a-induced leiomyoma shrinkage was due in part to a mechanism involving DNA damage (22) but not to apoptotic signal pathway initiated by Fas/Fas-L activity (9, 10). However, the questions on the molecular mechanism of DNA damage and its link to tissue ischemia or hormonal depletion as well as subsequent tumor degeneration still have no answers. In this article, we provide evidence on the expression of PARP, a nuclear protein playing a prominent role in the regulation of DNA repair and relevant gene transcription, to see how it reacts to GnRH-a effect.

The results revealed that the blood supply to uterine leiomyoma was geographically uneven, i.e. blood flow preferentially distributed to the peripheral part of tumor. The uneven blood supply to leiomyomas is in agreement with a study of Chiang et al. (27). The activation or up-regulation of PARP protein and the accumulation of PAR were more frequently detected in leiomyomas as compared with myometria. LA treatment further accentuated PARP production in leiomyomas, especially at the central portion of tumor. This was inversely correlated with the vascular supply to tumor tissue. Leiomyomas with high PARP and PAR grades following LA therapy were found to show greater tumor shrinkage. These results therefore support the findings that hyaline degeneration and geographic hydropic degeneration were found in a higher proportion of LA-treated patients (7) and further suggest to associate positively the degenerations of tumor with PARP and PAR grades. Moreover, in vitro studies pointed out that PARP expression had no dependence on hormonal depletion. Altogether, we suggest that it is not hormonal depletion but blood flow reduction that accounts for the tumor shrinkage after LA treatment.

Conventional color Doppler sonography examination could provide limited information regarding the qualitative and quantitative blood flow changes in a follow-up examination. In contrast, 3-D power Doppler sonography is more sensitive, less angle dependent, and not susceptible to aliasing. It is capable of the visualization of small vessels and the quantification of Doppler signal with a histogram software (28). Our power Doppler flow mapping indicated a progressive decrease in the blood flow to uterine leiomyoma following LA treatment. Because the blood supply was geographically partial to the peripheral region, the center of tumor would be more vulnerable to ischemia. Ischemia and the resultant oxidative stress can induce extensive subcellular damages. For instance, mitochondrial swelling is one of the common changes appearing in the ischemic cells (29) and observed in more than half of the uterine leiomyomas treated with GnRH-a (5). The swelling of mitochondria could not be reproduced in vitro by the deprivation of ovarian steroid hormones in culture medium, suggesting that hormonal depletion might not directly be responsible for the morphologic changes in mitochondria (30).

DNA is a target of oxidative stress prompted by ischemia. Impaired DNA strands can activate DNA repair and other protective mechanisms of the cell to maintain genome integrity (14, 15). In this study, the existence of PAR, indicating de novo PARP activation and NAD⁺ consumption, was a common phenomenon in uterine leiomyoma. In addition to activation, the up-regulation of PARP was also found prevalently in leiomyomas, especially in the central portion of the tumor. These results suggest that GnRH-a may cause a progressive decrease and deterioration of uneven blood supply to tumor centers and accentuate DNA damage of the cells in the regions. Activated PARP functions to catalyze poly(ADP-ribosyl)ation of nuclear proteins, which has been suggested to have some roles of importance in cellular functions, such as DNA synthesis, DNA repair, gene transcription, and cell differentiation (29). In addition to stimulating DNA repair, PARP is stated to be capable of modifying several transcriptional factors by poly(ADP-ribosylation) and preclude their binding to DNA (17, 31). Transcription of genes encoded in damaged DNA is thereby prevented, and those required for cell growth and proliferation also can be inhibited. Therefore, in the presence of sublethal DNA damage, increased PAR by GnRH-a treatment could have played roles in size reduction of leiomyoma cells.

In contrast, in the presence of severe DNA damage, PARP overactivation could deplete the intracellular stores of NAD⁺, in turn slowing the rate of glycolysis, mitochondrial electron transport, and ATP formation. The consequence is a severe energy crisis, leading to acute cell dysfunction and necrotic cell death. Therefore, despite playing an important role in DNA repair, PARP has long been recognized to induce cell death if overactivated under some conditions (18). Further evidence shows that genetic disruption or pharma-
cell nerosis. We have provided a molecular basis for uterine artery embolization (38) that is probably a similar but more aggressive mechanism as compared with GnRH-a-induced reduction of tumor blood flow. It offers a contribution for a better understanding on the real mechanism by which GnRH-a causes the degeneration of leiomyoma, which is the key of this therapy to manage the disease.

Acknowledgments

We thank Dr. Ko-Hung Lee for his assistance with the statistical analysis and discussion.

Received February 3, 2003. Accepted July 2, 2003.

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This work was supported by grants from the National Science Council, Taiwan (NSC90-2314-B-001-MY2, NSC90-2314-B-016-169); National Cheng Kung University Hospital (NCKUH 89-028, NCKUH 90-045); and MOE Program for Promoting Academic Excellent of Universities (91-B-FA09-1-4).

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