Augmented Serum Amyloid A1/2 Mediated by TNF-induced NF-κB in Human Serous Ovarian Epithelial Tumors

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INTRODUCTION

Ovarian cancer is the most common cause of cancer death from gynecologic malignancies and a heterogeneous disease (1). Epithelial ovarian carcinoma (EOC) is the most common type of ovarian cancer, accounting for 80–95% of all ovarian tumors (2). High-grade serous ovarian carcinoma (HGSOC) is the most common and
Serum Amyloid A in Ovarian Cancer

Reagents

Specific PCR primers for SAA1/2 and SAA4 were obtained from Eurofins MWG Operon (Huntsville, AL, USA). SYBR® Green Master Mix (cat #: 330503) and primers for TNF and GAPDH were purchased from SA Biosciences/Qiagen (Frederick, MD, USA) for qRT-PCR. Recombinant human TNF and lipopolysaccharide (LPS from Escherichia coli, 0111:B4) were obtained from R&D Systems (Minneapolis, MN, USA) and Sigma (St. Louis, MO, USA), respectively. The pGEM-T Easy Vector, pGL4.12 Luciferase Reporter Vector and the Luciferase Reporter Assay System were obtained from Promega (Madison, WI, USA). Lipofectamine, TRIZol, Moloney murine leukemia virus, SP6 and T7 RNA polymerase and all liquid culture media were acquired from Invitrogen (Grand Island, NY, USA).

Cell culture and treatments

The human ovarian cancer cell lines (OVCAR-3 and SKOV-3) were purchased from the American Type Culture Collection (Manassas, VA, USA). Ovarian cancer cells (~5×10⁴ cells/ml) were cultured in RPMI medium containing penicillin/streptomycin and 10% FBS at 37°C in a water-saturated atmosphere of 95% air and 5% CO₂. Treatments were initiated as outlined in Results.

Human high-grade serous ovarian epithelial tumor samples

The specimens (normal ovarian and tumor Stage IIIc tissues) in the study were obtained through the Cooperative Human Tissue Network (CHTN) and Institutional Review Board (IRB) approval at Vanderbilt University Medical Center.

Animals

C57BL6 mice (6 to 8-week-old, Charles River Laboratories, Wilmington, MA, USA) were given commercial pellet feed and drinking water ad libitum and housed with controlled 12-h light, 12-h dark cycle under pathogen-free conditions. All handling of animals and procedures were performed under institutional guidelines approved by the Institutional Animal Care and Use Committee at Meharry Medical College and adhere to the ARRIVE (Animal Research: Reporting In Vivo Experiments) guidelines for reporting animal research (16). LPS (100 μg/mouse) was injected intraperitoneally (ip) into the peritoneal cavity of mice and then the ovarian samples were collected at 2 h after LPS injection.

Reverse transcription polymerase chain reaction (RT-PCR) and real-time PCR

After isolating total RNA and eliminating genomic DNA, the expression of SAA1/2 and SAA4 were analyzed. Specific primers for SAA were designed as follows: 5’-TTT TCT GCT CCT TGG TCC TG-3’ (sense) and 5’-TGG AAG TAT TTG TCT GAG CCG-3’ (antisense) for human SAA1/2, 5’-CGT TCC AGG GTC TAT CTT
CAG-3′ (sense) and 5′-TTC CTC AGC TTT CTC GTT GG-3′ (antisense) for human SAA4. For RT-PCR, the reverse transcriptase reaction conditions, using random primers with Moloney murine leukemia virus, were at 42°C for 60 min followed by 94°C for 10 min. PCR was performed under the following conditions: denaturation at 94°C for 1 min, annealing at 60°C for 1 min and extension at 74°C for 1 min with 30 cycles. Amplified PCR products were analyzed by electrophoresis in 2% agarose gels containing 1 μg ethidium bromide/ml. The fluorescent images were photographed under UV light.

For real-time PCR, reverse transcription reactions were performed at 42°C for 15 min followed by 94°C for 5 min. According to manufacturers’ instructions, PCR was performed using a Bio-Rad CFX96 under the following two-step cycling program: 1 cycle at 95°C for 10 min, 40 cycles at 95°C for 15 sec and at 60°C for 1 min. Data analysis was performed using PCR Array Data Analysis Software (http://www.sabiosciences.com/RTPCR.php) provided by SABiosciences/Qiagen.

Construction of the SAA1 promoter and its deletion constructs
SAA1 (–490/+43) promoter was generated by PCR using primer sets as follows: 5′-GGG ATT ATA GGA GTG AGC CAC-3′ for sense and 5′-CTC CTC ACC TGA TCT GTG-3′ for antisense. The PCR was performed for 35 cycles at 94°C for 1 min, 40 cycles at 95°C for 15 sec and at 60°C for 1 min. The amplified SAA1 DNA fragment was subcloned into pGEM-T easy vector. Deletion constructs of pGL4.12 Luciferase Reporter Vector were generated from the SAA1 DNA fragment in pGEM-T Easy Vector under the same PCR conditions using the following primers containing the XhoI and Hind III sites: 5′-CTC GAG CCA GGG ACC ACA TCC AGC TTT-3′ for SAA1-P319, 5′-GCA CTC GAG CCA GGA ACT TGT CTT AGA CCG-3′ for SAA1-P139, and 5′-TAC CTC GAG CCA GGG ACC ACA TCC AGC TT-3′ for SAA1-P85. Common reversed primer sequence was 5′-CCG AAG CTT CTC ACC TGA TCT GTG CTG-3′ for SAA1+43. The constructs of the SAA1 promoter were confirmed by DNA sequencing analysis.

In situ hybridization
Nonradioactive methods for in situ hybridization on frozen sections were performed using digoxigenin-labeled SAA3 RNA probes (antisense and sense) as described previously (17,18).

RESULTS AND DISCUSSION

First, we evaluated the expression pattern of SAA isoforms in ovarian epithelial normal and tumor tissues. Identities of mRNAs (M23698 vs. M23700) and protein (AAI05797 vs. NP_110381) for SAA1 and SAA2 are 97% and 93%, respectively, by Align Sequences Nucleotide and Protein BLAST (https://blast.ncbi.nlm.nih.gov). Identities of mRNAs (M23698 vs. M23700) and protein (AAI05797 vs. EAW68415) for SAA1 and SAA4 are 69% (no significant similarity in megablast, but 69% in discontinuous megablast) and 55%, respectively. Because of difficult identification due to the highly similar sequences between SAA1 and SAA2, here we described as SAA1/2. We did not evaluate SAA3 isoform in human samples because human SAA3 is a pseudogene, unlike mouse SAA3, which is a main isoform (20). SAA1/2 mRNA was highly expressed in five different ovarian cancers compared to normal ovarian tissue samples (Fig. 1A). However, low expression of SAA4 mRNA was detected in tumor tissues compared to SAA1/2 (Fig. 1B). The results from RT-PCR confirmed the higher expression of SAA1/2 and similar detectable expression of SAA4 in the tumor tissues compared to normal tissues (Fig. 1C). These results suggest that SAA1/2 is the dominant isoform induced in human ovarian tumor tissues rather than SAA4.

Next, we investigated the expression of TNF in ovarian
Figure 1. SAA1/2 is a dominant isoform in human ovarian tumor tissues. (A) Expression levels of SAA1/2 mRNA determined by real-time PCR in five different ovarian normal (N1-N5) and tumor tissues (T1-T5). (B) Expression levels of SAA4 mRNA in normal ovarian tissues and tumor specimens determined by real-time PCR. (C) Visual confirmation of SAA1/2 and SAA4 expression by RT-PCR. After isolating total RNA, RT-PCR and real-time PCR were carried out using primers for SAA1/2 and SAA4. GAPDH was used as a loading control.

Figure 2. TNF enhances SAA1/2 expression in ovarian cancer. (A) Expression levels of TNF mRNA determined by real-time PCR in five different ovarian normal (N1-N5) and tumor tissues (T1-T5). (B) Correlation between mRNA levels of TNF and SAA isoforms. (C) Time-dependent effects of TNF on SAA1/2 mRNA by real-time PCR in ovarian cancer cell lines (OVCAR-3 and SKOV-3). Cells were incubated with TNF (10 ng/ml) for 0, 1, 3, 6, 12 and 24 hours. *Indicates significant increase (p<0.05) compared to its own control as calculated by the paired Student’s t test.
normal vs. tumor tissues and the correlation between TNF and SAA isoforms. TNF is well known to be a proinflammatory cytokine abundantly expressed in ovarian cancer (9-11). Interestingly, TNF is also a critical regulator for ovarian SAA (18,20). TNF was found to be highly upregulated in ovarian tumor tissues compared to normal tissues (Fig. 2A). Correlation between the expression levels of TNF and SAA isoforms revealed that SAA1/2 ($R^2=0.69$) was more closely associated with TNF rather than SAA4 ($R^2=0.33$) (Fig. 2B). Using TNF responsive ovarian cancer cells such as OVCAR-3 and SKOV-3 (6), we confirmed that TNF induced SAA1/2 mRNAs in a time-dependent manner (Fig. 2C). Because proinflammatory cytokines such as IL-1 and IL-6 were produced by ovarian tumors (10), these NF-κB activators also could augment ovarian SAA1/2 through NF-κB mediated signaling (18,20). In addition, TNF mRNA was ~4.63 fold highly expressed compared to IL-1α in human ovarian tumor tissues (data not shown).

We investigated whether TNF regulates human SAA1 at the promoter levels because SAA3, the main isoform of SAA in mouse, is induced by TNF (18,20). We generated a SAA1 promoter containing NF-κB like and consensus sites (Fig. 3A). We found that TNF induced human SAA1 promoter activity and IκB overexpression blocked TNF-induced promoter activity (Fig. 3B). These results implicate NF-κB mediated signaling in regulating human SAA1. In spite of the lack of NF-κB consensus sites in mouse SAA3 promoter, NF-κB like sites played a critical role in regulating SAA3 (18,20). Using deletion constructs, we examined which NF-κB site is critical for TNF-induced SAA1 promoter activity. SAA1-P401, P319 and P139 deletions were induced by TNF, whereas P85 without NF-κB site was not induced (Fig. 3C). These results indicate that the proximal NF-κB consensus site (~95~/86) is critical in regulating human SAA1.

Finally, because EOC is the most common type in ovarian cancer (2), we evaluated whether NF-κB activation induces the expression of SAA in ovarian epithelial cells exposed to LPS (21), a product known to stimulate release of TNF through toll-like receptors (TLRs), in mice. We injected LPS or vehicle (saline) to mice, collected the ovaries and assessed ovarian localization of SAA3 by in situ hybridization because SAA3 is a main isoform in mice (20). SAA3 mRNA was preferably localized in the ovarian epithelial cells and the thecal-interstitial layers as compared to granulosal cell layers (Fig. 4A). These results indicate that NF-κB signaling induces ovarian localization of SAA3 in ovarian epithelial cells.

Based on the NCBI GEO database (GSE51088), we compared expression levels of SAA1/2, SAA4, IL-1α, IL-1β, IL-6 and TNF in normal ovarian tissues (NOT), borderline ovarian tumor (BOT) and serous ovarian cancer (SOC). Along with TNF and LPS, IL-1β and IL-6

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**Figure 3.** TNF increases SAA1 promoter activity via NF-κB signaling. (A) DNA sequences of the human SAA1 promoter. (B) Effect of TNF and IκB on luciferase activity of SAA1 promoter in SKOV-3 cells. Results were normalized to the protein level and represented as a fold increase compared to empty vector (pA) controls. (C) Effects of TNF on luciferase activity of the SAA1 promoter with NF-κB site deletions. After SKOV-3 cells were transfected with SAA1 luciferase vectors overnight, cells were treated with TNF (10 ng/ml) for 6 h, followed by luciferase assay. Results were normalized to the protein level and represented as a fold increase compared to non-treated control. Grey and white colors indicate NF-κB like (lower case for unmatched DNA sequence) and consensus sites, respectively. *Indicates significant increase (p≤0.05) compared to its own control as calculated by the paired Student’s t test.
are also well-known NF-κB activators (21). Because the correlation coefficient in expression levels between SAA1 and SAA2 is 0.98, we described SAA1/2 together. SAA1/2, IL-1β and TNF were significantly expressed in SOC compared to NOT and BOT (Fig. 4B). The surface epithelium cells adjacent to the site of ovulation were associated with an increased risk of malignant transformation (10). Proinflammatory cytokines such as TNF, IL-1β and IL-6, produced by tumor itself and/or activated immune cells, have been shown to promote ovarian tumor growth and progression (4,5). Our results indicate that TNF is a key regulator of SAA1/2 via the NF-κB signaling pathway in EOC. A previous study showed that the expression of SAA in human EOC gradually increased during the progress through benign and borderline adenomas to primary and metastatic adenocarcinomas (15). In addition, high serum levels of SAA were found in patients with ovarian carcinoma, and the SAA levels are correlated strongly with the ovarian tumor marker CA-125 (15). Our analysis of the GEO dataset (Fig. 4B) support these previous results. The high expression of SAA1/2 via NF-κB mediated signaling in human EOC may have prognostic and therapeutic applications. In conclusion, the accumulation of SAA1/2 in EOC could be mediated by NF-κB activation through TNF, requiring further study regarding the functional role of SAA in EOC.

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CONFLICTS OF INTEREST

The authors have no financial conflicts of interest.

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