Induction of Serum Amyloid A Genes Is Associated with Growth and Apoptosis of HC11 Mammary Epithelial Cells

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In this study, we examined the expression and functions of serum amyloid A (SAA) isoforms during apoptosis of HC11 mammary gland epithelial cells. Expression of SAA mRNAs and apoptosis were increased in HC11 cells by serum withdrawal and gradually decreased upon the addition of serum, or epidermal growth factor (EGF). TNFα treatment of HC11 cells also induced expression of SAA genes, and the effect on SAA1 and SAA2 expression was suppressed by treatment with MG132, and in cells transfected with a dominant negative mutant form of IκBα. Similar results were observed in response to interleukin-1 (IL-1), IL-6 and interferon γ (IFNγ). Furthermore, overexpression of the SAA1 and SAA2 isoforms suppressed growth and accelerated apoptosis of HC11 cells by increasing caspase 3/7 and caspase 8 activities, but the apoptotic effect of tumor necrosis factor α (TNFα) on HC11 cells was not enhanced. We found that expression of SAA1 and SAA2, but not SAA3, was regulated by an NFκB-dependent pathway, and that overexpression of SAA isoforms accelerated the apoptosis of HC11 cells.

Key words: serum amyloid A; apoptosis; cytokines; NFκB; mammary epithelial cell

After completion of lactation, the mammary gland undergoes involution, regressing to a state resembling that of a virgin animal. This phase of mammary gland development is characterized by epithelial cell death and tissue remodeling. Previously, we identified several involution-induced genes, including SAA3, WDNM1, and sulfated glycoprotein-2, in bovine and mouse mammary glands.

SAA has been classified into two sub-families: acute-phase SAAAs (SAA1 and SAA2), which are dramatically up-regulated as acute-phase reactants during inflammation, and constitutive SAA, which is constitutively present in serum and is not significantly increased during inflammation. SAA1 and SAA2 are 95% homologous in both their coding and noncoding regions, and are coordinately induced in response to inflammation. In humans SAA1, 2, and 4 are found during the acute phase liver response in muscle cells, macrophages, and normal tissues. In the mouse, SAA1 and SAA2 are predominantly expressed in the liver, while SAA3 is present in a wide range of extra-hepatic cells and tissues, including macrophages, adipocytes, testis, and spleen. The physiological functions of the SAA family proteins include roles in cholesterol metabolism and transport, inhibition of lymphocyte and endothelial cell proliferation, induction of matrix metalloproteinases, and modulation of the inflammatory response via both anti- and pro-inflammatory activities.

Pro-inflammatory cytokines, such as IL-1β, IL-6, and
TNFα, trigger inflammation\textsuperscript{25} and stimulate the production of acute-phase proteins, such as c-reactive protein (CRP), SAA, and fibrinogen.\textsuperscript{26} Hence SAA is well-characterized as a member of a group of acute-phase proteins associated with many inflammatory diseases, but regulation of the expression and functions of extra-hepatic SAA family proteins has not been widely investigated.

In this study, we found that expression of SAA1 and 2, but not of SAA3, is regulated by an NFκB-dependent signaling pathway, and that overexpression of SAA genes accelerates the apoptosis of mammary epithelial cells.

\section*{Materials and Methods}

\textbf{Cell culture and hormone treatment.} The HC11 cell line, a clonal mouse mammary epithelial cell line derived from spontaneously immortalized COMMA1D epithelial cells,\textsuperscript{4–6} was cultured in growth medium at 37 \degree C in 5\% CO\textsubscript{2}. The growth medium (GM) consisted of RPMI 1640 (Gibco BRL, Carlsbad, CA) supplemented with 10\% fetal bovine serum (FBS; Gibco BRL), 1\% glutamine (Gibco BRL), 50\,μg/ml gentamycin (Gibco BRL), 5\,μg/ml insulin (Sigma, St. Louis, MO), and 10\,μg/ml EGF (Sigma). For differentiation, HC11 cells grown to confluence were incubated in differentiation medium (DM: 5\,μg/ml insulin, 5\,μg/ml prolactin, and 1\,μM dexamethasone) for the indicated number of days. The medium was changed every 48\,h. Hormone and cytokine treatments were carried out for the indicated times and at the indicated doses.

\textbf{Transfection of HC11 cells with expression vectors encoding SAA isoforms and \textit{IxB}α\textit{M}.} The plasmid pIRES-Neo (Clontech, Palo Alto, CA) and expression medium (DM: 5\times 10\textsuperscript{3} /C\textsubscript{14} TNFα, epithelial cells,\textsuperscript{4–6}) was cultured in growth medium at derived from spontaneously immortalized COMMA1D line, a clonal mouse mammary epithelial cell line isolated using a Qiagen endo free kit (Qiagen). Cells (pCMV-\textit{I}x\textit{B}α\textit{M} replaced with alanines, was purchased from Clontech) were transfected with recombinant \textit{cDNAs} encoding SAA isoforms and \textit{IxB}α\textit{M}. The recombinant plasmids were transfected into \textit{E. coli} cells, and the correct orientation of the SAA cDNAs in pIRES-Neo was confirmed by digestion with a QIAEX II gel extraction kit (Qiagen, Hilden, Germany). The \textit{SAA1}, \textit{SAA2}, and \textit{SAA3} cDNAs were then ligated downstream of the cytomegalovirus (CMV) immediate early promoter of pIRES-Neo. The recombinant plasmids were transformed into XLI-blue \textit{E. coli} cells, and the correct orientation of the SAA cDNAs in pIRES-Neo was confirmed by digestion with \textit{NolI} and sequence analysis. To block NFκB activation, a constitutively active form of \textit{IkB}α, in which serine residues 32 and 36 were replaced with alanines, was purchased from Clontech (pCMV-\textit{IxB}α\textit{M}). For transfection, plasmid DNA was isolated using a Qiagen endo free kit (Qiagen). Cells (3 \times 10\textsuperscript{5}) were seeded in 6-well plates in GM without antibiotics, and the next day they were transfected with recombinant plasmid (4\,μg) using lipofectamine 2000 (Gibco BRL), according to the manufacturer’s instructions. For the generation of stable cell lines, transfected cells were cultured in selection medium containing 600–1,000\,μg/ml Geneticin (G418; Gibco BRL). The medium was changed every 48\,h. After 14\,d of incubation in G418, single colonies were obtained by trypsinization within a cloning cylinder (Sigma). The cells were transferred into 24-well dishes and cultured in GM containing 1,000\,μg/ml of Geneticin. The cells were then transferred onto 6-well dishes for propagation. Genomic DNA was isolated from transfected cells using a Genomic DNA isolation kit (Promega, Madison, WI), according to the manufacturer’s instructions. To confirm the genomic integration of recombinant \textit{cDNAs}, PCR was performed using isolated genomic DNA as a template and primers specific for the CMV promoter 3\’ end region and the 5\’ end region of the \textit{cDNA}. PCR was carried out for 35 cycles (preheating at 94 \degree C for 5\,min; cycling at 94 \degree C for 1\,min 30\,s, at 55 \degree C for 1\,min 30\,s, and at 72 \degree C for 1\,min 30\,s; final elongation at 72 \degree C for 10\,min), and amplified fragments were analyzed by 1.0\% agarose gel electrophoresis. Expression of integrated cDNAs in stable transfectants was confirmed by reverse transcriptase (RT)-PCR.

\textbf{RT-PCR analysis.} Total RNA was extracted from mammary gland tissues at various reproductive stages and from HC11 cells using the Trizol reagent (Gibco BRL), according to the manufacturer’s instructions. Single-stranded cDNA was synthesized from 3\,μg of total cellular RNA using an oligo(dt) primer and reverse transcriptase, as recommended by the manufacturer (Perkin-Elmer/Cetus, Norwalk, CT). Reaction mixtures (20\,μl) were prepared as described above. The amplification program consisted of 24–36 cycles of the following parameters: 95 \degree C for 10\,s; annealing at 60 \degree C for 30\,s, and extension at 72 \degree C for 30\,s, followed by a final amplification step of 7\,min at 72 \degree C. We confirmed that the levels of the three different PCR targets (SAA1, SAA2, and SAA3) generated by 24–36 PCR cycles were in the linear range. The levels were analyzed and normalized as described previously (data not shown).\textsuperscript{27,28} The slopes of the amplification curves of the RT-PCR products were constant from 24 to 30 cycles, and products were quantified every other cycle in six independent experiments. The amplified products were separated by electrophoresis on a 1.2\% agarose gel, visualized with ethidium bromide, and analyzed by Multi-Analyst (Bio-Rad). The steady-state levels of SAA1, SAA2, and SAA3 and the \textit{GAPDH} mRNAs were quantified and normalized as described above. \textit{c-fos} and \textit{β-casein} were used as positive controls in cellular proliferation and in differentiation of mammary epithelial cells respectively. The primer sequences used in this study were as follows: SAA1 (accession no., NM009117), sense 5\’-ATGAAGGAAGCTAACTGGA-AAAATC-3\’, antisense 5\’-TCTCCTCAAGCAGTT-ACTACTGCAA-3\’; SAA2 (accession no., NM011314), sense 5\’-ATGAAGGAAGCTGCTGAAAGATG-3\’, antisense 5\’-TCTCCTCAAGCAGTTACTGCTC-3\’; SAA3 (accession no., NM 011315), sense 5\’-GCC-ACCATGAAGCTTCCATTGCCATATT-3\’, anti-
sense 5'-TCAGTATCTTTTAGGCCAGCCAGCAG-3'; c-fos, sense 5'-ATCGTCCGCTCTCATGTCGCCAA-3', antisense 5'-GCAACCCAGACTTCTCATTCTCA-3'; β-casein, sense 5'-GGTTACTCCAGCATCACGTCAC-3'; and GAPDH, sense 5'-TGCGAGCTCGAGTATGTTGTGCA-3', antisense 5'-CCACAAACAGGTTGCTGTATCCAA-3'.

Cell growth and apoptosis. To determine growth rates, transfected (vector, SAA1, SAA2, and SAA3) and non-transfected HC11 cells were plated in 6-well plastic tissue culture plates at a density of 1 x 10^5 cells/well. Twelve h after plating, the cells in one plate were incubated in a solution (2 ml/well) containing 0.5% crystal violet and 25% methanol, rinsed with deionized water, dried overnight, and resuspended in 100 μl citrate buffer (0.1 M sodium citrate in 50% ethanol) to assess plating efficiency. The remaining cultures were allowed to grow for an additional 1–4 d before staining. The intensity of crystal violet staining was assessed spectrophotometrically at a wavelength of 570 nm and quantified using Vmax Kinetic Microplate Reader and Softmax software (Molecular Devices, Menlo Park, CA). To determine apoptosis, cells were seeded in 96-well plates at a density of 1 x 10^3 cells/well and cultured in GM. On the following day, the cells were incubated in SFM for 12, 24, 36, and 48 h to induce apoptosis. The number of viable cells was measured using Trypan blue. DNA fragmentation analysis was performed as described by Herrman et al.,29 with some modifications. Briefly, genomic DNA was isolated using the Apoptotic DNA ladder kit (Roche, Mannheim, Germany), then subjected to 1% agarose gel electrophoresis to examine fragmentation. To determine the effect of overexpression of SAA isoforms on HC11 cell apoptosis, an Annexin V assay was performed according to the manufacturer's instructions (Annexin V-PE Apoptosis Detection Kit I; BD Biosciences, Palo Alto, CA). Briefly, cells grown in GM were shifted to SFM for 48 h, harvested by trypsinization, and pelleted by centrifugation. Next, 1 x 10^5 cells in 100 μl of 1X binding buffer were incubated with Annexin V-PE and 7-aminoactinomycin D, as directed. Flow cytometric analysis (FACStarPlus flow cytometer, Becton–Dickinson, Mountain View, CA) was performed to determine the number of apoptotic cells in each sample.

Caspase activity assays. Caspase 3/7 (Caspase-GLO 3/7 assay kit, Promega, Madison, WI) assays were performed according to the manufacturer’s recommendations.13 Briefly, vector- and SAA-transfected cells (pNeo, SAA1, SAA2, and SAA3, 5 x 10^5 cells per well) were plated in black 96-well dishes. After 48 h, the fluorescent caspase 3/7 substrate Z-DEVD-R110 was added to each well, and the plates were shaken gently at 500 rpm for 1 min, and then incubated at room temperature in the dark for 2 h. Samples were analyzed using a XFluor4 Ultra 384 fluorescence plate reader set at 485 nm (excitation)/535 nm (emission). Caspase-8 activity was measured using a Caspase-8 kit (TruPoint Caspase-8 Assay kit, Perkin Elmer) according to the manufacturer’s instructions. Briefly, cells were seeded in 100 mm diameter plastic tissue culture dishes and allowed to grow for 2 d. The cells were harvested by trypsinization and pelleted by centrifugation. The following incubation conditions were examined for each cell line: (i) IETD-7-amino-4-trifluoromethyl coumarin (AFC) (fluorescent caspase 8 substrate) alone; (ii) IETD-AFC plus the caspase 8 inhibitor IETD-fmk; and (iii) no substrate. All the samples and a standard curve of fluorescent-free AFC were incubated at 37°C for 2 h. Fluorescence was quantified using an XFluor4 Ultra 384 fluorescence plate reader (Tecan USA, Research Triangle Park, NC) at 405 nm (excitation)/508 nm (emission).

Statistical analysis. Where appropriate, levels of mRNA and protein expression, cell growth, apoptosis, and caspase activity data from in vitro studies were compared by Student’s t-test or ANOVA.

Results

First we examined the expression of SAA1, SAA2, and SAA3 mRNAs during mouse mammary gland development and involution by RT-PCR (Fig. 1A). We observed the highest level of expression of SAA3 mRNA on the 17th day of lactation, and low expression in early involution (day 1 of involution). However, SAA3 mRNA was not expressed during any other period of mammary gland development and involution. The expression patterns of SAA1 and SAA2 mRNAs were similar during mammary gland development and involution, expression first appearing in late pregnancy, reaching a maximum on day 17 of lactation, and then gradually decreasing up to day 4 of involution. These results indicate that SAA isoforms might be involved in the development and/or apoptosis of the mammary gland.

To examine the function of SAA isoforms, HC11 mouse mammary epithelial cells were used as an in vitro model system.4–6 HC11 cells were grown to confluence in growth medium (GM) containing 10% FBS and EGF for 2 d, and then cultured in differentiation medium (DM) and/or serum-free medium (SFM). In HC11 cells undergoing differentiation, expression of SAA1, SAA2, and SAA3 mRNAs was induced, and the levels of induction were maintained when the cells were switched to SFM (Fig. 1B and C). This pattern of SAA1, SAA2, and SAA3 expression was different from the expression pattern of β-casein, one of the markers of mammary gland differentiation. During culture in SFM, DNA fragmentation (Fig. 2B) and a dramatically decreased number of cells (Fig. 2A) were observed, confirming that the cells were undergoing apoptosis. Induction of SAA1, SAA2, and SAA3 mRNA levels was observed.
starting at 12 h of culture in SFM, and this high level of expression was maintained throughout the time period examined (Fig. 2C). SFM-mediated induction of SAA1, SAA2, and SAA3 mRNAs was abrogated by serum-stimulation (Fig. 2D and E). These results are in agreement with previous data showing that the expression of SAA3 increased during involution of the bovine mammary gland.30) To determine whether inhibition of apoptosis resulted in down-regulation of SAA1, SAA2, or SAA3 mRNA expression, HC11 cells were treated with EGF, a survival factor.6) SFM-mediated apoptosis of HC11 cells was abrogated by EGF treatment, as previously observed6) (Fig. 3A and F). These results provided further support for the thesis that the expression of SAA isoform mRNAs is regulated by apoptosis in HC11 cells.

TNFα is a pro-inflammatory cytokine that has been found to induce apoptosis in mammary epithelial cells.31) To determine whether expression of SAA isoform mRNAs was effected by TNFα, HC11 cells were treated with increasing doses (0–40 ng/ml) of TNFα under SFM conditions. The expression of SAA isoforms was increased by treatment with TNFα in a dose- and time-dependent manner (Fig. 4A–C). TNFα treatment also induced apoptosis of HC11 cells (Fig. 6H). To determine whether TNFα-induced expression of SAA isoform mRNAs was mediated by the nuclear factor (NFκB transcription factor, cells were incubated with carbobenzyoxy-L-leucyl-L-leucyl-L-leucinal (MG132), an inhibitor of NFκB, with or without TNFα, and the expression of SAA mRNAs was examined. Up-regulation of SAA1 and SAA2 expression by TNFα was strongly abrogated by MG132 (Fig. 5A). Expression of SAA3 was partially inhibited by MG132. To confirm this result, we developed stable cell lines overexpressing a constitutively active, dominant negative form of IκBα/IκBα (IκBαM) under the control of the CMV promoter, as well as control cells carrying the empty vector (pCMV). After 2 weeks of selection in G418, integration of plasmid DNA was confirmed by PCR using genomic DNA as the template and CMV50 and CMV30 primers. Expression of the IκBα gene was also confirmed by RT-PCR (data not shown). Stable transfectants were grown to confluence in GM, maintained for 2 d in medium containing 2% FBS but lacking insulin and EGF, and then incubated in SFM in the presence or absence of TNFα. Similarly to the results of MG132 treatment, up-regulation of SAA1 and SAA2 expression by TNFα was abrogated in IκBα-overexpressing HC11 cells, but not in control cells (Fig. 5B.
and C). Also consistently with the effect of MG132 treatment, up-regulation of SAA3 expression by TNFα was not changed in IκBα-overexpressing HC11 cells (Fig. 5B and C). We expanded this analysis using other cytokines that have been found to induce SAA gene expression in hepatic and non-hepatic cells through the action of NFκB.26 Similarly to TNFα, IL-1, IL-6, and IFNγ also induced the expression of SAA mRNAs. The induction of SAA1 and SAA2 mRNAs by IL-1, IL-6, and IFNγ was completely inhibited in IκBα-overexpressing HC11 cells, but not in pCMV-expressing control cells (Fig. 5D), while the induction of SAA3 expression by IL-1, IL-6, and IFNγ did not change in IκBα-overexpressing HC11 cells (Fig. 5D).

SAA is a member of a group of acute-phase proteins that are associated with many inflammatory diseases, but the function of the SAA proteins has not been fully elucidated. To gain a better understanding of the function of the SAA proteins in the proliferation and apoptosis of mammary epithelial cells, expression vectors encoding SAA1, SAA2, and SAA3 under the control of the CMV promoter were constructed using pCMV-IREs, and then recombinant plasmids were transfected into HC11 cells. Stable cell lines overexpressing SAA1, SAA2, and SAA3, as well as control cells transfected with pNeo, were isolated after 2 weeks of culture in G418 selection medium. We isolated at least 10 colonies from each transfection, and integration of plasmid DNA was confirmed by PCR using genomic DNA and IRES 5' and IRES 3' primers. Expression of
SAA1, SAA2, and SAA3 was confirmed by RT-PCR, and the colonies with the highest levels of expression of each isoform were selected for further experiments (Fig. 6A). Stable transfectants were cultured in GM, and the growth of each cell line was compared with vector-transfectants (control cells). SAA1- and SAA2- but not SAA3-transfected cells exhibited delayed growth (1.24 ± 0.18, 1.59 ± 0.23, and 2.19 ± 0.21 respectively) as compared to control cells after 4 d (2.32 ± 0.36, Fig. 6B). The very delayed growth rate of SAA1 and SAA2-transduced HC11 cells indicates a role for SAA isoforms in HC11 proliferation. It was difficult to distinguish, however, whether the growth inhibition induced by overexpression of SAA isoforms that we observed in vitro was due to an effect on cell cycle arrest or rather to apoptosis. Increased apoptosis can lead to a decrease in the measured proliferation rate by increasing cell loss and reducing the number of viable cells produced over time. To determine the mechanism of growth inhibition of the SAA isoforms, we measured apoptosis directly in HC11 cells that overexpressed the SAA isoforms. Cells were cultured in GM as above, then switched to SFM, and cell viability was examined by trypan blue exclusion assay. Cell viability had decreased significantly by 1.4–2.1 fold in SAA1-, SAA2-, and SAA3-transfected cells (36 ± 4%, 35 ± 1%, 53 ± 1.5% respectively) as compared to the control cells (75 ± 4.3%) at 48 h of SFM treatment (Fig. 6C). Furthermore, DNA fragmentation...
was initially observed in SAA1-, SAA2-, and SAA3-transfected cells after 12 h of incubation in SFM, but was not detected in the control cells (Fig. 6D). To clarify further the effect of overexpression of SAA isoforms on apoptosis, cells were pretreated as above, and then cultured in SFM, and apoptosis was examined by flow cytometry. Apoptosis was significantly increased by 2.2–3.1 fold in SAA1-, SAA2-, and SAA3-transfected cells (55/C6:4% 41/C6:6% 45/C6:1% respectively) as compared with the control cells (18/C6:1%) at 48 h of incubation in SFM (Fig. 6C). Taken together, these results suggested that overexpression of SAA1 and SAA2 but not of SAA3 leads to delayed HC11 cell growth in GM culture conditions and to accelerated apoptosis under SFM culture conditions, and that SAAs possibly exert their primary effect on apoptotic signaling pathways.

To determine the mechanism by which SAAs stimulate the basal rate of apoptosis in HC11 cells, we examined the activity of several caspases in SAA stable transfectants. First we measured caspase 3/7 basal activity in SAA-transfected cells and control cells by assaying the cleavage of the fluorescence-conjugated caspase 3/7 substrate, ZDEVD-R110.32) Overexpression of SAA1, SAA2, and SAA3 had a significant effect on caspase 3/7 substrate cleavage (4.2-, 3.9-, and 2.5-fold increases respectively) relative to control, vector-transfected cells (Fig. 6F). When we assayed a different fluorescent substrate specific for caspase 8 (IETD-AFC), we found that this upstream initiator caspase was also affected by SAA expression. Consistently with caspase 3/7 activity, overexpression of SAA1, SAA2, and SAA3 induced strong increases in caspase 8 activity (6.8-, 4.9-, and 2.7-fold increases compared to control respectively; Fig. 6G). These results imply that the increased basal rate of apoptosis in cells constitutively expressing SAA isoforms was due to activation of caspases in these cells. TNFα-induced apoptosis under SFM conditions was not significantly enhanced in SAA1-, SAA2-, or SAA3-transfected cells, compared to the absence of TNFα treatment (51 ± 4.9% vs. 55 ± 6.5%, 38.7 ± 2.9% vs. 42 ± 5.9%, and 46.5 ± 5.1% vs. 47.2 ± 4.2% respectively; Fig. 6E). In contrast, there was a significant increase in apoptosis in vector-transfected control cells in the presence of TNFα as compared to treatment with vehicle alone (59.6 ± 4.6% and 19.8 ± 2.1% respectively).

**Discussion**

SAA proteins are acute-phase proteins expressed primarily in the liver. SAA levels are closely related to the activity of chronic inflammatory diseases,33) and...
recently were found to be a predictor of coronary artery disease. The mouse SAA family consists of SAA1, SAA2, and SAA3. SAA1 and SAA2, known as acute-phase SAA, dramatically increase, by as much as 1,000-fold, during inflammation. SAA3 is classified a constitutive SAA (C-SAA). In the mouse, extra-hepatic synthesis of SAA2 has been definitively documented in the kidney and intestines, while that of SAA1 appears to be restricted to the kidney. SAA3, however, is produced in a wide range of tissues and cell types, including macrophages.

Many studies have focused on the regulation of SAA gene expression in liver cells and its putative function as a modulator of acute-phase infection and inflammation. The regulation of expression of SAAs in mammary epithelial cells is less well understood. Previously, SAA3 was identified as a gene that was up-regulated during mammary gland involution. We have found that in high density cultures, serum starvation induces apoptosis of HC11 cells. In the current study, we found that the same condition results in elevated expression of SAAs (Fig. 2). Decreased apoptosis of HC11 cells in SFM culture conditions was observed with the re-addition of serum, or EGF treatment, with a concomitant decrease in the expression of SAAs (Figs. 2–4). These results indicate that expression of SAA mRNAs is closely related to the apoptosis of HC11 cells.

The pro-inflammatory cytokines IL-1, IL-2, IL-6, IFNg, and TNFα regulate SAA gene expression in...
hepatic and non-hepatic cells, either alone or in various combinations. Evidence is also accumulating that apoptosis induced by these cytokines involves the activation of downstream signaling pathways (i.e., activation of NFκB). NFκB regulates the transcription of many target genes that are effectors of immune, inflammatory, and acute phase responses. An analysis of the promoters of SAA1 and SAA2 indicated that NFκB is essential for cytokine-induced gene expression in HepG2 and Hep3B cells. In agreement with these data, our results indicate that TNFα, IL-1, IL-6 and IFNγ induced expression of SAA mRNAs.
in mammary epithelial cells. Also, TNFα-mediated induction of SAA1 and SAA2 expression, but not of SAA3, was strictly dependent on NFκB, since inactivation of NFκB by either an NFκB-specific inhibitor (MG132) or overexpression of a dominant negative IkBα mutant abrogated TNFα-mediated induction of SAA1 and SAA2 expression. NFκB, however, is unlikely to be directly involved in stimulating SAA3 expression by TNFα or other cytokines.

A recent study found that human SAA3, originally identified as a pseudogene, is expressed in the human mammary gland, and that expression of SAA3 is induced by prolactin (PRL) or lipopolysaccharide (LPS) in human mammary epithelial cells. The promoter region of human SAA3 contains putative STAT3 (TTC-CCGGAAA) and STAT5-like (TTCCCGGAA) binding sites positioned 1,607 bp upstream (bp) and 429 bp upstream of the 5′UTR respectively. These cis-acting elements have been implicated in the up-regulation of SAA3 transcription following LPS or PRL stimulation. PRL-induced activation of STAT5 in human mammary epithelial cell lines has been demonstrated. IL-6 activates the JAK/STAT pathway in human mammary epithelial cells, but the influence of TNFα on the activation of STAT factors in human mammary epithelial cells is currently unknown. Bing et al., however, reported that NFκB was involved in the regulation of SAA3 promoter activity during cytokine-mediated induction in HepG2 cells. This apparent discrepancy might be due to cell-specific differences in signaling pathways and transcription factors. Hence the involvement of alternate signaling pathways, in addition to NFκB activation, in the up-regulation of SAA3 expression by TNFα and other cytokines warrants further study. Among the molecules implicated in the regulation of SAA3 expression, NFκB, Oct-1, AP-1, STAT3, and STAT5 also appear to be involved in the apoptosis of mammary epithelial cells. Although the precise biological functions of the SAA proteins has not been determined, several studies suggest that the N-terminal region of the various SAA isoforms is in part responsible for their structural and functional properties. The N-terminal region of SAA1 and SAA2 specifically binds to and transports cholesterol into HepG2 and aortic smooth muscle cells, indicating a possible role of A-SAA1 and A-SAA2 in the modulation of cholesterol flux during an acute phase response, as well as in atherosclerosis. Other studies indicate that the N-terminal region of A-SAA is the precursor for amyloid A, a chief constituent of amyloid fibrils found in secondary amyloidosis. Recently, Larson et al. isolated the SAA3 isoform from the colostrum of several mammals and found that pretreatment of human intestinal epithelial cells with a synthetic peptide consisting of the N-terminal 10 amino acids of M-SAA3 stimulates production of the protective intestinal mucin MUC3 and significantly reduces enteropathogenic Escherichia coli (EPEC) adherence. More recently, SAA was reported to induce IL-8 secretion and to inhibit platelet aggregation. SAA levels are closely related to chronic inflammatory and coronary artery diseases. SAA functions pathologically as a precursor of amyloid A (AA) protein in AA (secondary) amyloidosis, a serious complication of chronic inflammatory diseases such as rheumatoid arthritis (RA), juvenile inflammatory arthritis, and Castleman disease. In the current study, our results suggest that overexpression of SAAs in HC11 cells accelerates apoptosis by enhancing caspase activity. Caspases are a family of proteases involved in proteolytic cleavage of cellular proteins during apoptosis, and many studies have shown that apoptosis is accompanied by the activation of specific caspases. Caspases 3 and 7 are effector caspases, and their activation by upstream apical or initiator caspases (such as caspase 8) is one of the final events before cell death. The results of the current study suggest that TNFα-induced apoptosis of HC11 cells is mediated by increased expression of SAAs, since overexpression of SAAs accelerated the apoptosis of HC11 cells under SFM conditions and the effect of TNFα-mediated apoptosis was not further enhanced in SAA overexpressing HC11 cells.

In summary, we found that overexpression of SAA1, SAA2, and SAA3 suppresses the growth of mammary epithelial cells under GM conditions and accelerates apoptosis through the activation of caspase activity under serum starvation conditions. Furthermore, induction of apoptosis in SAA-overexpressing HC11 cells was not further enhanced by TNFα treatment, suggesting that TNFα-mediated induction of apoptosis is mediated through induction of the expression of SAA isoforms. Additional studies are needed to identify the molecular components of the apoptotic pathway induced by overexpression of SAA genes.

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