A novel pathogenic role of the ER chaperone GRP78/BiP in rheumatoid arthritis

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An accumulation of misfolded proteins can trigger a cellular survival response in the endoplasmic reticulum (ER). In this study, we found that ER stress–associated gene signatures were highly expressed in rheumatoid arthritis (RA) synoviums and synovial cells. Proinflammatory cytokines, such as TNF and IL-1β, increased the expression of GRP78/BiP, a representative ER chaperone, in RA synoviocytes. RA synoviocytes expressed higher levels of GRP78 than osteoarthritic (OA) synoviocytes when stimulated by thapsigargin or proinflammatory cytokines. Down-regulation of Grp78 transcripts increased the apoptosis of RA synoviocytes while abolishing TNF- or TGF-β–induced synoviocyte proliferation and cyclin D1 up-regulation. Conversely, overexpression of the Grp78 gene prevented synoviocyte apoptosis. Moreover, Grp78 small interfering RNA inhibited VEGF-induced angiogenesis in vitro and also significantly impeded synoviocyte proliferation and angiogenesis in Matrigel implants engrafted into immunodeficient mice. Additionally, repeated intraarticular injections of BiP-inducible factor X, a selective GRP78 inducer, increased synoviocyte proliferation and angiogenesis in the joints of mice with experimental OA. In contrast, mice with Grp78 haploinsufficiency exhibited the suppression of experimentally induced arthritis and developed a limited degree of synovial proliferation and angiogenesis. In summary, this study shows that the ER chaperone GRP78 is crucial for synoviocyte proliferation and angiogenesis, the pathological hallmark of RA.
Figure 1. ER stress response is increased in RA synovia and synovial cells. (A) Illustration of protein processing in the ER pathway with up-regulated genes in RA synovial tissue. Node colors represent fold change in RA synovial tissues as compared with normal synovial tissues (red, up-regulated; and green, down-regulated). (B) Functional enrichment analysis of up-regulated genes in OA tissues compared with normal. (C and D) Representative
ER stress is a cellular danger signal, which is triggered by a failure to fold newly synthesized ER proteins. Diverse conditions including hypoxia and low glucose, which are frequently observed in the RA joints (Stevens et al., 1991), may act as an ER stress to the cells, although evidence of this is lacking. Unless two compensatory mechanisms of unfolded protein response (UPR) and ER-associated degradation (ERAD) work properly, ER stress causes cell damage and eventually cell death (Rutkowski and Kaufman, 2004; Schröder and Kaufman, 2005). It has become apparent that dysregulated UPR plays an important role in the pathogenesis of some human diseases (Kaufman, 2002; Pfaffenbach and Lee, 2011) such as diabetes mellitus and Alzheimer’s disease, in which affected tissues are devoted to extracellular protein synthesis (Katayama et al., 2004). RA shares a common feature with these disease conditions, in that synoviocytes and other inflammatory cells produce large amount of proteins like cytokemokinines and matrix metalloproteins that perpetuate arthritic conditions.

GRP78/BiP is a molecular chaperone with antiapoptotic properties and a central regulator of ER homeostasis (Lee, 2007; Pfaffenbach and Lee, 2011). GRP78 promotes tumor proliferation, survival, metastasis, and resistance to a wide variety of therapies (Lee, 2007; Dong et al., 2008; Pfaffenbach and Lee, 2011). The discovery of GRP78 expression on the cell surfaces of cancer cells has led further to the development of new therapeutic approaches to cancer (Lee, 2007; Pfaffenbach and Lee, 2011). Rheumatoid synovium can be viewed as a local tumor because synoviocytes, the principal components of the pannus, proliferate abnormally in RA joints in a manner reminiscent of tumors; for example, they resist apoptosis and also exhibit other features of metastatic cancer cells (Firestein, 1996; Müller-Ladner et al., 1996; Okamoto et al., 2000). Therefore, it can be postulated that abnormal GRP78 response to ER stress contributes to synoviocyte hyperplasia and leads to bone and cartilage destruction in RA, although the exact role of ER stress in the pathophysiology of RA remains to be determined.

We demonstrate herein that ER stress–associated gene signatures are highly expressed in RA synovia and synovial macrophages. In cultured synoviocytes, proinflammatory cytokines, such as TNF–α and IL–1β, increased GRP78 expression. Downregulation of Gsp78 transcripts using small interfering RNA (siRNA) increased the apoptosis of RA FLSs while abolishing TNF–α–or TGF–β–induced FLS proliferation and cyclin D1 upregulation. Moreover, Gsp78 siRNA blocked VEGF165–stimulated angiogenesis in vitro and impeded synoviocyte proliferation and angiogenesis in Matrigel implants that were engrafted into immunodeficient mice. On the contrary, BiP-inducible factor X (BIX), a selective GRP78 inducer, prevented the apoptosis of FLSs and increased the progression of experimental osteoarthritis (OA) in mice, mimicking RA pathology. Moreover, heterozygous deficiency of the Gsp78 gene resulted in a limited degree of inflammatory cell infiltration, angiogenesis, and synovial hyperplasia and prevented the progression of experimental arthritis in mice. Collectively, our results suggest that GRP78 is crucial for the survival and proliferation of RA synoviocytes and angiogenesis, which suggests that the ER chaperone GRP78 response contributes to the pathogenesis of RA.

RESULTS
Expression of ER stress–associated genes in RA synovia and synovial cells
To demonstrate that ER stress response is activated in RA synovium, we first investigated global gene expression profiles in the synovial tissues of RA patients. We analyzed three synovial tissue–derived microarray datasets published previously in the GEO DataSets database (available under accession nos. GSE1919, GSE7307, and GSE12021). As shown in Fig. 1 A, differentially expressed genes (DEGs) between RA and normal subjects were identified using integrative statistical hypothesis testing (Bolstad et al., 2003; Hwang et al., 2005; see Materials and methods). From the KEGG pathway database, protein processing in ER pathways, including ER nuclear signaling, ER protein recognition (e.g., GRP78/HSPA5), ERAD, and ER protein targeting pathway, was significantly enriched by DEGs up-regulated in RA synovium (Fig. 1 A). However, unlike RA synovium, there was no statistical difference in above ER stress–associated pathways between OA and normal synovi ums (Fig. 1 B). Immunohistochemical analysis was also performed to examine the expressions and localizations of ER stress marker proteins in the synovial tissues of three RA patients and three OA patients. The expression of GRP78, a well–established ER stress marker, was observed in all three RA synovial tissue sections at high levels. Positive staining was predominantly seen in the lining layer of the hyperplastic synovium (Fig. 1 C). GRP78 was also expressed in the lining layer of OA synovium (Fig. 1 C), but its expression level was relatively weak compared with that in RA synovium. Moreover, RA synoviums exhibited higher expression levels of other ER stress–associated molecules, immunohistochemical stainings for ER stress–associated molecules in synovium sections obtained from RA and OA patients. (C) Tissues stained with anti–GRP78 antibody are representative of three RA and three OA patients. Intense staining in the synovium was observed in the lining layer (arrowheads). (D) Immunohistochemical staining of RA and OA synovia using anti–anti-DIIT3 (CHOP), anti–p–IRE1, anti–ATF6, and anti–XBP1 antibody. Bars, 120 µm. (E) Heat map displaying 32 ER stress–associated up-regulated genes in RA macrophages. (F) Cellular processes enriched by DEGs in RA macrophages. Functional enrichment analysis of up-regulated DEGs was performed using DAVID software. (G) Quantitative real-time PCR assays of a subset of DEGs in RA macrophages. *, P < 0.05 versus normal macrophages. (I and I) Data show mean ± SD.
including DDIT3 (CHOP), IRE1, ATF6, and XBP-1 (spliced and total) in the lining layer and/or sublining leukocytes as compared with OA synoviums (Fig. 1 D).

We also analyzed ER-associated gene expression profiles in synovial cells isolated from RA joint fluids. Fig. 1 E shows the DEGs between RA synovial macrophages (n = 6) and normal macrophages derived from peripheral blood monocytes (n = 2). Of the 616 up-regulated DEGs found in RA synovial macrophages, the heat map shows 32 DEGs associated with ER stress processes. Three cellular processes, including response to oxidative stress, response to ER stress, and the ER–nuclear signaling pathway, were significantly enriched by 32 DEGs using DAVID software (Fig. 1, E and F). Using real-time PCR analysis, we also confirmed that independent RA macrophages (n = 5) exhibited higher messenger RNA (mRNA) expression levels of 10 selected DEGs and proximal UPR genes, including CHOPATF6, and the spliced form of XBPI (xXBPI), than independent normal (n = 4) and OA macrophages (n = 9; Fig. 1 G). Moreover, basal levels of GRP78 and p-eIF2α protein were also higher in RA macrophages than normal macrophages (Fig. 1, H and I). Collectively, ER stress–associated gene signatures were highly expressed in synoviums and synovial cells of RA patients.

**GRP78 expression is induced in synoviocytes by proinflammatory cytokines**

In addition to synovial macrophages, FLSs are a major component of the lining layer and invasive pannus. To determine whether FLSs are also involved in ER stress responses, we analyzed the mRNA expression profiles of primary RA (n = 3) and OA (n = 3) FLSs. Interestingly, unlike in freshly isolated RA macrophages, the aforementioned ER processes were not enriched by DEGs up-regulated in RA FLSs (not depicted). Western blot analysis revealed that GRP78, IRE1, XBP1, and eIF2α expression levels were similar in RA (n = 8) and OA (n = 8; not depicted) FLSs, which contrasts with the results in synovial tissues and macrophages (Fig. 1). We postulated that favorable culture conditions from passage 3 to 8, during which the medium contained high growth factors and culture medium remains to be defined. Therefore, we investigated whether GRP78 regulates cell survival in RA FLSs. Several ER stress–associated proteins, including GRP78, ATF-6, BCL-2, and caspase-12, are involved in cell survival or death (Kaufman, 2002; Rutkowski and Kaufman, 2004; Schröder and Kaufman, 2005). As shown in Fig. 3 A, thapsigargin treatment increased the expressions of GRP78, p-eIF2α, ATF-6, and caspase-12 in rheumatoid synoviocytes but decreased BCL-2 expression. To examine the role of GRP78 in the survival of RA FLSs, we introduced Grp78 siRNA into the RA FLSs. As shown in Fig. 3 B, Grp78 knockdown rendered FLSs more susceptible to apoptosis in the presence or absence of sodium nitroprusside (SNP). Recently, by screening chemical libraries, Kudo et al. (2008) identified a novel molecule Bix/GRP78 inducer X (BIX) that selectively induces GRP78. It has been reported that BIX pretreatment reduces ER stress–induced cell death by preferentially increasing GRP78 expression in neuroblastoma cells (Kudo et al., 2008; Oida et al., 2008). As shown in Fig. 3 C, BIX increased GRP78 protein expression in RA FLSs and protected RA FLSs from the cell death induced by thapsigargin. Additionally, overexpression of the Grp78 gene blocked tunicamycin-, thapsigargin-, or SNP-induced apoptotic death of RA FLSs (Fig. 3, D–G), indicating that GRP78 inhibits FLS death. Moreover, when treated with thapsigargin or tunicamycin, RA FLSs showed less apoptosis than OA FLSs, as assessed by MTT assay, DNA fragmentation ELISA, and the APOPercentage apoptosis assay (not depicted), which is in accord with our results on inducible GRP78 expression (Fig. 2, E–G) and with a previous study (Yamasaki et al., 2006). The aforementioned findings suggest that intracellular GRP78 plays a critical role in maintaining synoviocyte survival against proapoptotic ER stress conditions in joints.

**GRP78 is a regulator of synoviocyte survival**

ER chaperones like GRP78 serve as a class of regulators of cell survival. GRP78 protects against ER stress–induced apoptosis in a wide variety of tissues and organs, as well as in cancer cells (Lee, 2007; Dong et al., 2008; Pfaffenbach and Lee, 2011). However, the role of GRP78 in FLS death under conditions of ER stress remains to be defined. Therefore, we investigated whether GRP78 regulates cell survival in RA FLSs. Several ER stress–associated proteins, including GRP78, ATF-6, BCL-2, and caspase-12, are involved in cell survival or death (Kaufman, 2002; Rutkowski and Kaufman, 2004; Schröder and Kaufman, 2005). This allowed us to identify stimuli that mimic or trigger ER responses in FLSs. In inflamed joints, rheumatoid synoviocytes are exposed to various proinflammatory cytokines, such as IL-1β and TNF, and hypoxia (Feldmann et al., 1996). Thus, an experiment was conducted to determine the effect of the proinflammatory cytokines on the GRP78 expression in FLSs because GRP78 is known to be the central regulator of ER stress (Lee, 2007; Pfaffenbach and Lee, 2011). GRP78 expression in the RA FLSs increased up to 48 h after stimulation with 1 ng/ml IL-1β or 10 ng/ml TNF (Fig. 2 A). Furthermore, GRP78 was also found to be induced by 100 µM CoCl2 treatment, which simulates hypoxic conditions (Fig. 2 A). In contrast, 10 ng/ml IL-10, an antiinflammatory cytokine, failed to increase GRP78 expression, whereas 20 µg/ml tunicamycin instigated it in the same cells (Fig. 2 B). Both IL-1β and TNF induced other ER stress response genes, including CHAC1, EIF2AK2, ERO1L, DERL1, DERL3, PPP1R15A, DDIT3, CCND1, SELS, EIF2AK3, CREB3L2, COL4A3BP, BCL2, ATP2A1, and ATF6, in RA FLSs (Fig. 2, C and D). In addition, thapsigargin or tunicamycin time–dependently increased GRP78 protein expression in both RA and OA synoviocytes, but these increases occurred to a greater extent in RA cells than in OA cells (Fig. 2, E and F). Moreover, Grp78 mRNA expression levels in RA FLSs, stimulated with IL-1β, TNF, TGF-β, CoCl2, or thapsigargin, were significantly higher than those in stimulated OA FLSs, indicating that RA FLSs hyperrespond to these cytokines and other ER stresses (Fig. 2 G). Collectively, these results suggest that RA synoviocytes exhibit increased GRP78 response to proinflammatory cytokines and hypoxia, which induces hyper-UPR of RA FLSs.
control (scrambled) siRNA (Fig. 4 A). The number of RA FLSs stimulated with TGF-β or TNF was also markedly reduced by Grp78 siRNA transfection (Fig. 4 B). The regulation of cyclin D1 expression represents an important step for the control of cellular proliferation in response to growth factors (Terada et al., 1999). As shown in Fig. 4 C, cyclin D1 levels in RA FLSs were time-dependently increased by TNF or TGF-β treatment. The down-regulation

Figure 2. Induction of GRP78 in synoviocytes by proinflammatory cytokines. (A and B) Regulation of GRP78 expression in RA FLSs by proinflammatory cytokines. Cells were stimulated with 10 ng/ml TNF, 1 ng/ml IL-1β, 10 ng/ml IL-10, or 100 µM CoCl2 for the indicated times. 20 µg/ml tunicamycin was used as a positive control. GRP78 levels in FLSs were determined by Western blot analysis. The data shown are representative of more than four experiments with similar results. (C and D) Quantitative real-time PCR assays of ER stress response genes induced by TNF (C) or IL-1β (D) in RA FLSs. Cells were stimulated with 10 ng/ml TNF or 1 ng/ml IL-1β for 48 h. Fold inductions were calculated using the 2^ΔΔCt method. Data show mean ± SD. (E and F) RA (n = 4) and OA (n = 4) FLSs were treated with 10 µM thapsigargin (E) or 20 µg/ml tunicamycin (F) for the indicated times. GRP78 expression was determined by Western blot analysis. In the lower panel, the optical density ratio of GRP78/β-actin (β-act) expression is presented as the mean values of four separate experiments. (G) Comparison of Grp78 mRNA expression in OA (n = 6) versus RA (n = 6) FLSs, as determined by real-time PCR. Cells were stimulated with 1 ng/ml IL-1β, 10 ng/ml TNF, 10 ng/ml TGF-β, 100 µM CoCl2, or 5 µM thapsigargin (Tg) for 8 h. Bars show mean and SEM. *, P < 0.05 versus OA FLSs.
Effect of GRP78 on VEGF-induced angiogenesis

In addition to the abnormal proliferation of synoviocytes, increased angiogenesis is a critical step in the establishment and progression of RA (Koch, 1998; Firestein, 1999). In a previous study, partial reduction of GRP78 in Grp78+/− heterozygous mice and in mice with conditional Grp78 knockout in endothelial cells substantially reduced tumor...
Reduction of synoviocyte proliferation by the down-regulation of Grp78 transcripts in vivo

To confirm the effect of GRP78 on synoviocyte proliferation in vivo, we subcutaneously implanted Matrigel plugs containing RA FLSs transfected with Grp78 siRNA into immunodeficient mice in the presence or absence of TGF-β (Fig. 6, A and B). Before Matrigel implantation, we first confirmed that the Grp78 down-regulatory effect persisted at 7 d after Grp78 siRNA transfection into RA FLSs (Fig. 6 A). We then counted the number of RA FLSs implanted and endothelial cells recruited in Matrigels placed in mice for 7 d in the presence or absence of TGF-β. Without TGF-β, the number of cells positive for human HLA class I, indicating RA FLSs, was significantly lower in Matrigel implants containing Grp78 siRNA than in those with control siRNA (Fig. 6, A and B). Before Matrigel implantation, we first confirmed that the Grp78 down-regulatory effect persisted at 7 d after Grp78 siRNA transfection into RA FLSs (Fig. 6 A). We then counted the number of RA FLSs implanted and endothelial cells recruited in Matrigels placed in mice for 7 d in the presence or absence of TGF-β. Without TGF-β, the number of cells positive for human HLA class I, indicating RA FLSs, was significantly lower in Matrigel implants containing Grp78 siRNA than in those with control siRNA (Fig. 6, A and B). When 50 ng/ml TGF-β was added to implants (Fig. 6, C and D), the number of HLA + cells increased in both Grp78 siRNA- and control siRNA-transfected gels, but it was greater in control gels (2.4-fold for control siRNA vs. 1.5-fold for Grp78 siRNA), indicating that Grp78 siRNA mitigates synoviocyte proliferation in vivo. It has been shown that human RA FLSs induce angiogenesis through a VEGF-mediated pathway in immunodeficient mice (del Rey et al., 2009). In parallel with this, we found that Grp78 down-regulation in FLSs resulted in a decrease in the extent of angiogenesis in Matrigel implants.

microvessel densities (Dong et al., 2011). Furthermore, knockdown of Grp78 using siRNA suppressed human microvascular endothelial cell proliferation and increased apoptosis (Dong et al., 2011). In agreement, we found that Grp78 siRNA nearly completely inhibited VEGF165-stimulated human umbilical vein endothelial cell (HUVEC) proliferation (Fig. 5, A and B) but did not affect cell viability until 48 h after transfection, as determined by the MTT assay (not depicted). Under nonlethal time conditions, we then tested whether Grp78 siRNA regulates tube formation by endothelial cells. As was expected, VEGF165-induced tube formation was markedly reduced in the presence of the Grp78 siRNA (Fig. 5 C). During angiogenesis, endothelial cells migrate in response to several chemotactic factors. Therefore, we attempted to determine whether GRP78 affects VEGF165-induced HUVEC migration. As shown in Fig. 5 D, Grp78 siRNA suppressed VEGF165-induced wound migration of HUVECs without decreasing cell viability, as determined 24 h after transfection. Furthermore, VEGF165-induced HUVEC chemotaxis in a Boyden chamber was also blocked by transfection with Grp78 siRNA for 24 h but was not affected by control siRNA (Fig. 5 E). Collectively, these results indicate that GRP78 directly mediates VEGF165-induced migration/chemotaxis and endothelial cell proliferation.
BIX increases synovial hyperplasia and angiogenesis in mice with collagenase-induced OA

We then examined the pathological role of GRP78 in vivo. To this end, BIX was injected periarticularly into the joints of mice with collagenase-induced OA every other day for 2 or 4 wk. Both synovial proliferation and bone erosion were rarely detected in joints of mice injected periarticularly with BIX alone (Fig. 7 A), indicating that the injection procedure itself did not cause joint pathology nonspecifically. Synoviocyte proliferation was only modest in vehicle-treated OA mice as well (Fig. 7 A). However, BIX plus collagenase–treated mice showed a marked increase in synoviocyte proliferation in joints and exhibited cartilage and bone damage mimicking rheumatoid synovitis (Fig. 7 A). As a whole, BIX plus collagenase–treated mice showed significantly higher degrees of synovial hyperplasia and pannus formation than vehicle plus collagenase–treated mice (n = 6 per each group; Fig. 7 B). In addition, vWF staining of joints revealed new vessel formation with synoviocyte hyperplasia in BIX plus collagenase–treated mice but not in control mice (Fig. 7 C). Moreover, immunohistochemical staining for proliferating cell nuclear antigen (PCNA) revealed that the number of positive synoviocytes was much higher in BIX plus collagenase–treated mice (n = 6) than in control mice (n = 6; Fig. 7 D), demonstrating that BIX increased synoviocyte proliferation. These results show that repeated injections of BIX cause synoviocyte proliferation and angiogenesis in mice with collagenase-induced OA.

Decreased arthritis severity in Grp78 heterozygous mice

Finally, we attempted to determine the role played by GRP78 in vivo in animal model of RA. To this end, experimental arthritis was induced in mice with reduced GRP78 by injection of anti–type II collagen antibody. In this passive model of arthritis, the innate immune system, including macrophages, FLSs, and endothelial cells, plays a central role in the progression of joint disease independent of adaptive immunity (Ji et al., 2002). Because complete Grp78 deficiency proved lethal, we
DISCUSSION

GRP78/BiP is a stress protein of the hsp70 family and is involved in the regulation of ER homeostasis. GRP78 level is widely used as a sentinel marker of ER stress under pathological conditions (Lee, 2007; Pfaffenbach and Lee, 2011). GRP78 has also been suggested to be a putative autoantigen in RA (Bläss et al., 2001; Corrigall et al., 2001). GRP78-specific antibodies were found to be present in 63% of RA patients, in 7% of patients with other rheumatic diseases, and in no healthy subjects (Bläss et al., 2001). Mice with collagen-induced arthritis have been shown to produce high levels of anti-GRP78 antibodies (Corrigall et al., 2001). T cell responses to GRP78 have been reported to be elevated in RA patients, particularly in joints (Corrigall et al., 2001). In one study (Corrigall et al., 2001), although GRP78 injected into mice failed to induce experimental arthritis, it completely inhibited the development of arthritis when given before injecting type II collagen, which suggests that exogenous GRP78 has immune tolerogenic potential that could be useful for treating RA (Panayi and Corrigall, 2008). In addition, there may be a link between the enhanced activation of the GRP78 in plasma cells and the local production of GRP78.
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We provide the first evidence that expression levels of ER stress–associated genes, including GRP78, BCL2, CCND1, HSPA1B, DNAJB11, HYOU1, PDIA6, ATP2A2, DNAJB1, EIF2AK3, DIT3, and sXBP1, were higher in RA macrophages (n = 5) than in OA tissue macrophages (Fig. 1 G); only EROL1 mRNA expression was comparable between RA and OA macrophages. These data clearly show that RA macrophages contain a high level of UPR genes, which may contribute to elevated ER signatures in RA synoviums.

Some evidence demonstrates cross talk between the inflammatory response and ER stress response (Xue et al., 2005; Zhang and Kaufman, 2008). For example, in murine fibrosarcoma cells, the proinflammatory cytokine TNF was found to trigger UPR, increasing the expressions of XBP1 and GRP78 and the phosphorylation of eIF2 (Xue et al., 2005). IL-1 and TNF also have been reported to induce ER stress in hepatocytes, leading to the activation of CREBH, a transcription factor which mediates acute phase response in liver (Zhang et al., 2006). In the present study, we demonstrated first that IL-1 and TNF up-regulated GRP78 expression in RA synoviocytes, which indicates that proinflammatory cytokines act as ER stressors in RA joints. Moreover, RA FLSs showed a greater GRP78 response to proinflammatory cytokines (IL-1β, TNF, or TGF-β), CoCl2, or thapsigargin than OA FLSs. Considering that RA synoviums contain high numbers of inflammatory cells (e.g., macrophages) compared with OA synoviums, higher levels of ER signature in RA synoviums could be just a result of higher numbers of macrophages expressing UPR genes. To address this issue, we freshly isolated OA macrophages from OA synovial tissues (n = 9), immediately obtained mRNA from the cells, and then determined the expression of ER stress–related genes by real-time PCR analysis. We provide the first evidence that expression levels of ER stress–associated genes, including GRP78, BCL2, CCND1, HSPA1B, DNAJB11, HYOU1, PDIA6, ATP2A2, DNAJB1, EIF2AK3, DIT3, and sXBP1, were higher in RA macrophages (n = 5) than in OA tissue macrophages (Fig. 1 G); only EROL1 mRNA expression was comparable between RA and OA macrophages. These data clearly show that RA macrophages contain a high level of UPR genes, which may contribute to elevated ER signatures in RA synoviums.

During monocyte to macrophage differentiation, GRP78 expression can be increased (Dickhout et al., 2011). Because RA synoviums contain high numbers of inflammatory cells (e.g., macrophages) compared with OA synoviums, higher levels of ER signature in RA synoviums could be just a result of higher numbers of macrophages expressing UPR genes. To address this issue, we freshly isolated OA macrophages from OA synoviums (n = 9), immediately obtained mRNA from the cells, and then determined the expression of ER stress–related genes by real-time PCR analysis. We provide the first evidence that expression levels of ER stress–associated genes, including GRP78, BCL2, CCND1, HSPA1B, DNAJB11, HYOU1, PDIA6, ATP2A2, DNAJB1, EIF2AK3, DIT3, and sXBP1, were higher in RA macrophages (n = 5) than in OA tissue macrophages (Fig. 1 G); only EROL1 mRNA expression was comparable between RA and OA macrophages. These data clearly show that RA macrophages contain a high level of UPR genes, which may contribute to elevated ER signatures in RA synoviums.

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Evidence is also emerging that GRP78 protects many tissues and organs under ER stress (Wang et al., 2010; Pfaffenbach and Lee, 2011). The present study shows that GRP78 mediates TNF- or TGF-β–induced synoviocyte proliferation, regulating cyclin D1 expression in vitro. In a xenotransplantation model, we also found that GRP78 plays a role in synoviocyte proliferation in vivo. It is well known that IL-1β, TNF, and TGF-β levels are greater in the sera, synovial fluid, and inflamed synovial tissues of RA patients than in those of OA patients (Feldmann et al., 1996). Therefore, the synoviocytes of RA patients may have a greater opportunity to be stimulated by these cytokines than the OA synoviocytes. Under these circumstances, proinflammatory cytokines, such as TNF and IL-1β, induce GRP78 expression in RA FLSs (Fig. 2, A–D), and this increased level of GRP78, in turn, could lead to synovial hyperplasia, which facilitates the further secretion of cytokines and thus establishes a vicious cycle. The suggestion of cross talk between ER stress and chronic inflammation was bolstered by the recent finding that IRE1 activates the NF-κB and JNK pathway, leading to the production of TNF (Hu et al., 2006).

If a cell fails to maintain ER homeostasis, the UPR will initiate apoptosis to protect the organism by removing stressed cells that produce misfolded or malfunctioning proteins (Zhang and Kaufman, 2008). Several pathways have been implicated in ER stress–induced apoptosis, and the full induction of apoptosis seems to require the concomitant activations of several death signals, including CHOP, eIF2α, ATF6, and caspase-12 (Kaufman, 2002; Rutkowski and Kaufman, 2004; Schröder and Kaufman, 2005). In this study, we showed that thapsigargin treatment increased the expression of GRP78, p-eIF2α, ATF6, and caspase-12 while decreasing BCL-2 expression. Moreover, the down-regulation of Grp78 transcripts using siRNA was found to increase apoptosis of RA FLSs. On the contrary, GRP78 overexpression prevented FLSs from apoptotic death induced by an ER stressor or SNP. Given a higher GRP78 response to ER stress in RA FLSs than in OA FLSs, our data suggest that ER hyperresponsiveness may confer apoptotic resistance on rheumatoid synoviocytes under ER stress conditions, leading to synovial hyperplasia.

GRP78 promotes survival in both proliferating and dormant tumors (Reddy et al., 2003; Li and Lee, 2006; Lee, 2007) and induces the proliferation and invasion of cancer cells (Reddy et al., 2003; Misra et al., 2005, 2006; Pyrko et al., 2007). Evidence is also emerging that GRP78 protects many tissues and organs under ER stress (Wang et al., 2010; Pfaffenbach and Lee, 2011). The present study shows that GRP78 mediates TNF– or TGF-β–induced synoviocyte proliferation, regulating cyclin D1 expression in vitro. In a xenotransplantation model, we also found that GRP78 plays a role in synoviocyte proliferation in vivo. It is well known that IL-1β, TNF, and TGF-β levels are greater in the sera, synovial fluid, and inflamed synovial tissues of RA patients than in those of OA patients (Feldmann et al., 1996). Therefore, the synoviocytes of RA patients may have a greater opportunity to be stimulated by these cytokines than the OA synoviocytes. Under these circumstances, proinflammatory cytokines, such as TNF and IL-1β, induce GRP78 expression in RA FLSs (Fig. 2, A–D), and this increased level of GRP78, in turn, could lead to synovial hyperplasia, which facilitates the further secretion of cytokines and thus establishes a vicious cycle. The suggestion of cross talk between ER stress and chronic inflammation was bolstered by the recent finding that IRE1 activates the NF-κB and JNK pathway, leading to the production of TNF (Hu et al., 2006).
Evidence is emerging that a subfraction of GRP78 can be expressed on the cell surface, particularly under pathophysiologic conditions (Ni et al., 2011). Cell surface GRP78 has been implicated in cell signaling regulating proliferation and viability distinct from its chaperone function in the ER. Indeed, binding of cell surface GRP78 with various ligands triggers multiple biological processes in different cell types. Furthermore, surface GRP78 is a potential receptor for targeted cancer therapy in cancer and chronic vascular disease (Sato et al., 2010). For example, binding of activated α2-macroglobulin to GRP78 induces the UPR and cancer cell proliferation (Misra et al., 2006). Interaction of GRP78 with cell surface T-cadherin promotes endothelial cell survival (Philippova et al., 2008). In a recent study, surface-expressed citrullinated GRP78 on monocytes/macrophages was found to bind anti-CCP antibodies and stimulate TNF production (Lu et al., 2010). We also found that GRP78 was expressed in the surface of FLSs as well as in the ER (unpublished data). Although binding partners of GRP78 on FLSs have not been identified, surface GRP78 could play proinflammatory, pro-survival, and proliferative roles by association with its functional ligands that might exist in the RA joints, as it does in cancer and endothelial cells (Misra et al., 2006; Philippova et al., 2008; Ni et al., 2011).

Angiogenesis is a critical event in the formation and maintenance of invasive pannus in RA, especially during the early disease stages (Koch, 1998; Firestein, 1999). The initiation of angiogenesis is known to be associated with the up-regulation of many angiogenic factors, and in particular, VEGF165 plays a crucial role in neovascularization during pannus formation (Koch, 1998; Firestein, 1999). VEGF165 levels are elevated in the serum, synovial fluid, and inflamed synovium of RA patients (Lee et al., 2001), and these levels have been shown to be highly correlated with RA disease activity (Lee et al., 2001). In this study, we demonstrate for the first time that VEGF165-induced angiogenesis is dependent on GRP78. Under nonapoptotic conditions, Grp78 siRNA was found to repress VEGF165-induced proliferation, tube formation, wound migration, and the chemotaxis of endothelial cells, indicating that GRP78 directly controls VEGF165-induced angiogenesis. In contrast, RA FLSs are the major source of several angiogenic factors, including VEGF, in joints (Koch, 1998; Firestein, 1999). We found that knockdown of the Grp78 transcript in RA FLSs significantly impeded the recruitment of endothelial cells to Matrigel implants engrafted into immunodeficient mice. These data, together with previous studies (Koch, 1998; Firestein, 1999), suggest that GRP78-mediated FLS proliferation increases angiogenic factor production and that this indirectly contributes to neovascularization and pannus formation.

We also found that repeated injections of BIX, a selective inducer of GRP78, into the joints of mice with experimental OA promoted synovial hyperplasia, bone erosion, and new vessel formation in synovia. Furthermore, PCNA+ proliferating cells were also found to be markedly increased by BIX. Conversely, mice with Grp78 haploinsufficiency (Grp78<sup>−/−</sup>) showed only limited synovial proliferation histologically, decreased angiogenesis, and lower levels of experimentally induced arthritis. These observations demonstrate that GRP78 is crucial to the development of chronic arthritis, involving synoviocyte proliferation and angiogenesis in vivo. Interestingly, repeated injections of BIX alone did not affect the joint pathology. This suggests that increased GRP78 expression alone is not sufficient to establish an RA pathology, which might require additional stimuli to sustain abnormal UPR, such as proinflammatory cytokines and hypoxia. Nevertheless, it is remarkable to observe that a 50% reduction in the GRP78 activity resulted in a significant suppression of arthritis in vivo. Collectively, our work provides the first evidence that normal synovium may be converted to a rheumatoid pathology when it is repeatedly exposed to ER stress to instigate GRP78 expression and that, conversely, genetic ablation of Grp78 prevents the development of arthritis in mice.

In addition to UPR, ERAD is also required to avoid ER stress in the cells (Yamasaki et al., 2005). The UPR relieves ER stress by inducing genes such as ER chaperones to increase the protein-folding capacity of the ER, as well as by inhibiting general protein translation. In contrast, ERAD eliminates misfolded or unassembled proteins that accumulate in the ER through the ubiquitin–proteasome system (Yamasaki et al., 2005). Recently, synoviolin, an E3 ubiquitin ligase for ERAD, was identified in RA FLSs and synovial lining tissue (Amano et al., 2003). Heterozygous synoviolin knockout mice are protected against collagen-induced arthritis development associated with reduced synovial hypercellularity (Amano et al., 2003). The aforementioned studies on synoviolin (Amano et al., 2003; Yamasaki et al., 2005) are common to our results in that dysregulated ER response critically contributes to synovial hyperplasia and the development of chronic arthritis. Thus, it would be interesting to investigate whether two biological processes, UPR and ERAD, affect each other to induce RA.

In summary, RA joints were found to contain the genetic signatures of ER stress. GRP78, a central regulator of ER response, was highly expressed in RA synoviums and synoviocytes and conferred apoptotic resistance on RA synoviocytes. GRP78 controlled synoviocyte proliferation and angiogenesis in vitro. The critical roles played by GRP78 in synoviocyte proliferation and angiogenesis were confirmed in Matrigel implants and in an animal model of arthritis, which suggests that ER chaperone GRP78 contributes to the pathogenesis of RA by inducing synovial hyperplasia and angiogenesis. These findings provide new insights of the role of GRP78 in the pathogenesis of RA and explain how normal synoviocytes develop an aggressive phenotype in RA joints. Thus, we suggest that the regulation of ER stress response offers a new treatment target for chronic autoimmune arthritis.

**MATERIALS AND METHODS**

**Patients.** 38 patients who fulfilled the 1987 revised criteria of the American Rheumatism Association for RA (Arnett et al., 1988) were studied. The mean age of the RA patients (8 males and 30 females) was 53.4 ± 10.2 yr. The mean disease duration was 63.4 mo (range 6–145 mo). 26 (68.4%) of 38 RA patients were positive for rheumatoid factor, and 29 patients (76.3%)
were positive for anti-CCP antibody. All RA patients were treated with disease-modifying antirheumatic drugs (DMARDs), including methotrexate (52.6%), antimarial drugs (65.7%), sulfasalazine (31.5%), bucillamine (13.2%), and anti-TNFα antibody (7.9%). Comparisons were made with 35 patients with OA (7 males and 28 females) and 12 healthy controls (3 males and 9 females) who had no rheumatic diseases. The mean ages of the OA patients and healthy controls were 54.4 yr and 51.6 yr, respectively. No difference was found in age and sex between RA and OA or healthy controls. The study protocol was approved by the Institutional Review Board of the Catholic Medical Center (VCMC08BR067). All patients gave written informed consent to the study protocol.

Isolation of FLSs, endothelial cells, and synovial fluid macrophages. FLSs were obtained from the synovial tissues of RA or OA patients as described previously (Yoo et al., 2006) and incubated in DMEM supplemented with FCS (Invitrogen) or with insulin-transferrin-selenium A (ITSA; Life Technologies). In some experiments, mouse FLSs were isolated from the joints with FCS (Invitrogen) or with insulin-transferrin-selenium A (ITSA; Life Technologies). Isolation of FLSs, endothelial cells, and synovial fluid macrophages. Normal synovial tissues were obtained using an enhanced chemiluminescent technique. Resulting films were scanned, and optical densities were quantified using Quantity One 1-D software (Bio-Rad Laboratories). Experiments were repeated three to four times for separate samples.

Immunohistochemical staining. 5-μm sections of paraffin-embedded synoviums of RA and OA patients were treated with pepsin for 30 min and blocked with BSA for 30 min at room temperature. Tissue sections were then incubated with antibodies to GRP78 (1:100; Stressgen Bioreagents), CHOP (1:100; Santa Cruz Biotechnology, Inc.), p-eIF2α (1:100; Abcam), ATF-6 (1:100; Santa Cruz Biotechnology, Inc.), and XBP-1 (1:100; Abcam) overnight at 4°C. Isotype control antibody (Sigma-Aldrich) was used as a negative control. Each slide was washed three times in TBS and incubated with biotinylated anti–mouse IgG (Vector Laboratories) in a humid chamber for 30 min. GRP78-positive cells were detected using peroxidase-conjugated streptavidin (Vector Laboratories) followed by 3′3-diaminobenzidine tetrahydrochloride (DAB; Vector Laboratories). The slides were counterstained with hematoxylin.

FACS analysis of GRP78. Surface expressions of GRP78 on FLSs of RA or OA patients were detected by flow cytometry. In brief, cells were detached with 1 mM EDTA in PBS and stained with DyLight 488–conjugated anti–GRP78 antibody (Abcam) for 1 h at 4°C in the dark. DyLight 488–conjugated IgG2a isotype antibody (Abcam) was used as a control. Fluorescence was measured by the FACSCanto II system (BD), and the data were analyzed using FlowJo 8.7.3 software (Tree Star).

Cloning and overexpression of the human Grp78 gene. The human Grp78 gene was amplified by PCR from cDNA obtained from rheumatoid synoviocytes using two pairs of primers encompassing the Grp78 coding region (262–2226). The forward primers encoded an XhoI site, and the reverse primers contained a HindIII restriction site: Grp78 forward primer, 5′-CCTCGAGATGAAGCTCTCCCTG-3′; and Grp78 reverse primer, 5′-AAGGTTTCAACTACATCTTTTTC-5′. PCR was performed over 35 cycles of 94°C for 30 s, 55°C for 60 s, and 72°C for 60 s. The resulting PCR product was cloned into pFLAG-CMV2 vector (Sigma-Aldrich) to generate the pFLAG-hGrp78 construct, which was verified by sequencing (ABI PRISM 310; Applied Biosystems). The plasmid DNA transfection of the hGrp78 gene into SV40-immortalized rheumatoid synoviocytes was conducted in 6-well plates using Lipofectamine 2000 reagent (Yoo et al., 2006).

Cell viability: MTT assay. Synoviocyte viability was determined by an MTT assay as described previously (Lee et al., 2006; Kong et al., 2010).

Detection of apoptosis. FLS apoptosis level were determined using the APOPercentage Apoptosis Assay kit (Biocolor Ltd; Kong et al., 2010). Digital images of APOPercentage dye–labeled cells, which appear bright pink against a white background under a light microscope, were used to quantify apoptotic cell numbers. Fold increases in apoptosis level were expressed as pixel numbers. Degrees of apoptosis were also determined using cellular DNA fragmentation ELISA (Roche) as described previously (Lee et al., 2006).

Down-regulation of Grp78 transcripts. For the down-regulation of Grp78 transcripts, RA FLSs were transfected with Grp78 siRNA (Santa Cruz Biotechnology, Inc.) using Lipofectamine 2000 (Invitrogen). Scrambled RNAs were provided by Santa Cruz Biotechnology, Inc. Grp78 mRNA...
and protein expression levels in FLs were determined by RT-PCR and by Western blot analysis using anti-GRP78 monoclonal antibody (Abcam), respectively. To detect Gp78 mRNA, RT-PCR was performed using the following Gp78-specific primers: sense, 5′-GCTCGACTCGAATTC-CAAG-3′; and antisense, 5′-TTGTCAAGGTCCTTACC-3′. PCR amplification was conducted over 25 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 60 s, and elongation at 72°C for 30 s. GAPDH mRNA expression was used as an internal control.

**Synovioctye proliferation assay.** After knocking down of Gp78 mRNA, RA FLs were subjected to a proliferation assay based on [3H]thymidine incorporation as described previously (Lee et al., 2006; Yoo et al., 2006). Proliferative responses were expressed as counts per minute in stimulated FLs as compared with unstimulated cells. Analysis was performed in triplicate, and results are expressed as the mean ± SD.

**Wound migration and capillary tube formation assays.** Wound migration of HUVECs was measured as described previously (Lee et al., 2006; Yoo et al., 2006). In brief, HUVECs plated to confluence on 60-mm culture dishes were wounded with pipette tips and then treated with 20 ng/ml VEGF165 in M199 medium supplemented with 0.5% FCS for 12 h. Migration was quantified by counting cells that had moved beyond a reference line. To determine capillary tube-forming ability, HUVECs were seeded on a layer of previously polymerized Matrigel (BD) containing 20 ng/ml VEGF and incubated for 12 h. Tube-forming ability was quantified by measuring the lengths of tubes in five randomly chosen low-power fields (×50) per well using Image-Pro Plus version 4.5 software (Media Cybernetics).

**Assay of endothelial cell chemotaxis.** The chemotactic migration of HUVECs was assayed using a transwell chamber equipped with a 6.5-mm-diameter polycarbonate filter of pore size 8 μm. In brief, 50 ng/ml of recombinant VEGF in DME containing 1% FCS was placed in lower wells, and HUVECs suspended in DME containing 1% FCS at a final concentration of 5 × 10^4 cells/ml were placed in upper wells. The chamber was then incubated at 37°C for 12 h. Nonmigrating cells on the upper surfaces of filters were removed by wiping with a cotton swab, and migrated cells were fixed and stained with a Diff-Quik kit (Baxter Diagnostics). Chemotaxis was quantified by counting cells that migrated to the lower side of the filters at ×200. Eight random fields were counted for each assay.

**Matrigel plug assay in immunodeficient mice.** 500 µl Matrigel containing 5 × 10^4 RA FLs was injected subcutaneously into the abdomen of athymic nude mice (The Jackson Laboratory) as described previously (del Rey et al., 2009). After 7 d, skins were pulled back to expose the Matrigel plugs, which remained intact. Plugs were embedded in paraffin and stained using hematoxylin and eosin or used for immunolabeling experiments. Sections were stained with anti–mouse vWF antibody (Abcam) and anti–human HLA class I antibody (Abcam). Sections were counterstained with hematoxylin. Quantitative data were obtained by counting the number of positively staining cells per area in digitalized images covering the whole Matrigel area under confocal microscopy.

**Effect of BIX on synoviocyte proliferation in mice with experimental OA.** Male C57BL/6 mice ( Orient), aged 8–12 wk, were used in all experiments. BIX, a selective GRP78 inducer (Kudo et al., 2008; Oida et al., 2008), was synthesized and purified at the Korea Research Institute of Chemical Technology. Experimental OA was induced as previously described (Yoo et al., 2007). In brief, 1 U of type VII collagenase from Clostridium histolyticum (Sigma-Aldrich) was injected twice on days 0 and 2 into the right knee joints of mice. The right knee joints of negative control mice were injected twice with the same volume (5 µl) of PBS. From day 2, 20 µg BIX was administered to experimental mice percutaneously every other day for 2 or 4 wk. Control mice received vehicle alone. Histological assessment of joint injuries was performed at 2 or 4 wk after the primary collagenase injection. Immunohistochemical staining for vWF was also performed in knee joints using the anti-vWF antibody (Abcam). Degrees of synovial hyperplasia, pannus formation, and joint destruction were determined using a scoring protocol, whereby severity was scored on a scale of 0–4, where 0 = absent, 1 = minimal, 2 = mild, 3 = moderate, and 4 = severe.

**Induction of anticollagen antibody-induced arthritis.** 8-wk-old heterozygous Gp78^+/− mice, backcrossed for eight generations onto the C57BL/6 background, and their wild-type littermates (Gp78^+/+) were used for this study. 5 mg anti-type II collagen antibody (Chondrex) was injected intravenously into the two groups of mice, and 3 d later, 50 µg LPS was administered intraperitoneally (Kong et al., 2010). The clinical severity of arthritis was graded as described previously (Kong et al., 2010). During the course of arthritis, footpads and hind foot swelling were also measured daily using a microcaliper. The paw thickness index was defined as the diameter of an arithmetic ankle divided by its diameter on day 0 and was expressed as a percentage (Kong et al., 2010). On day 10, paws and ankles were harvested. Degrees of inflammation, synovial hyperplasia, and joint destruction were determined using a standard scoring protocol as previously described (Kong et al., 2010).

**Statistical analysis.** Data are expressed as the mean ± SD. Comparisons of the numerical data between groups were performed by the paired or unpaired Mann-Whitney U test. P-values <0.05 were considered statistically significant.

**Online supplemental material.** Table S1 shows the gene-specific primers used for real-time PCR. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.201111783/DC1.

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