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Caspase-11 Promoter-GFP Construct as a Dual Reporter of Cytotoxicity and Inflammation

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Abstract: Caspase-11 has been known as a dual regulator of apoptosis and inflammatory response. An unusual feature of caspase-11 is that its expression is induced by apoptotic or proinflammatory stimuli. Utilizing these unusual features of caspase-11, we have developed a simple and sensitive assay method to screen pro- or anti-apoptotic/inflammatory molecules. To develop this assay method, we generated a reporter construct where GFP expression is regulated by caspase-11 promoter. When several types of cultured cells were transfected with this reporter construct and subsequently treated with various apoptotic or proinflammatory molecules, expression of GFP by the activation of caspase-11 promoter was easily detected by fluorescence microscopy or spectrofluorometry. In addition, a reduction of the GFP fluorescence was detected when an agent reported to suppress caspase-11 induction was applied. These results suggest that our reporter system can be used to screen pro- or anti-apoptotic/inflammatory molecules.

Key words: Caspase-11 promoter-GFP, cytotoxicity, inflammation

As libraries of chemical compounds expand, a great many useful small molecules are waiting to be screened for their useful bioactivities. In addition, as the screening and utilization of novel chemical compounds and natural products accelerates, a development of more effective and sensitive bioassay systems is required to evaluate their possible toxicity. To meet these needs, we have developed a simple and sensitive tool by which both anti- and pro-apoptotic/inflammatory molecules can be screened. This method is based on the fact that caspase-11 is an inducible caspase and it is a dual regulator of apoptosis and inflammatory response (Wang et al., 1998; Kang et al., 2000; 2002). We constructed a reporter system where green fluorescence protein (GFP) cDNA is fused to caspase-11 promoter. If cultured cells are transfected with this construct, the cells will produce fluorescent protein upon receiving apoptotic or proinflammatory stimuli. This reporter system can be used to screen both pro- and anti-apoptotic/inflammatory molecules.

Caspase-11 is a murine caspase with very interesting features. Unlike other caspases, transcription of caspase-11 is strictly regulated. Caspase-11 is not expressed in healthy tissues but its expression is dramatically upregulated under various pathological conditions such as brain ischemia (Kang et al., 2000), septic shock (Wang et al., 1996; Kang et al., 2002), experimental autoimmune encephalomyelitis (Hisahara et al., 2001), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced model of Parkinson's disease (Furuya et al., 2004) and myocardiac ischemia-reperfusion injury (Wang et al., 2005). Many studies reported upregulation of caspase-11 expression in cultured cells by various apoptotic or inflammatory drugs such as etoposide (Kang et al., 2000), LPS (Kobori et al., 2004), sodium valproate (Phillips et al., 2003), benzene (Yoon et al., 2003), and ethanol (Kang et al., 2005). When induced, 43 and 38 kDa caspase-11 bands are detected by immunoblot assay. It is possible that the 38 kDa caspase-11 is a product of alternative translation since point mutation of the second methionine into alanine abolished the expression of the 38 kDa caspase-11 only (SJ Kang, unpublished observation).

Induction of caspase-11 has been reported to be regulated by NF-κB in response to LPS (Schauvliege et al., 2002; Kobori et al., 2004) or by signal transducer and activator of transcription 1 (STAT1) in response to interferon-γ (Schauvliege et al., 2002). The caspase-11 promoter identified by Schauvliege et al. (2002) spans about 1 kb in the 5' upstream of caspase-11 transcription start site. Putative transcription factors for the caspase-11 include

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NF-κB, STAT1, CREB, NFAT, SP-1 and IRF (Schuvliege et al., 2002). A precise mechanism of caspase-11, however, remains to be determined.

The types of caspase-11 expressing cells are also varied. Expression of caspase-11 has been observed in neurons (Kang et al., 2000), astrocytes (Suk et al., 2003), oligodendrocytes (Hisahara et al., 2001), microglia (Suk et al., 2003), embryonic fibroblasts, L929, BALB/c 3T3 cells (Wang et al., 1996; Kobori et al., 2004), B and T cells (Kang et al., 2002), macrophages (Schauvliege et al., 2002) and hepatoma cells (Phillips et al., 2003).

Another interesting feature of caspase-11 is that it can regulate both apoptosis and inflammatory response. In general caspases can be grouped into two; one that regulates apoptosis and the other that regulates inflammatory response. Caspases known to regulate inflammatory response are caspase-1, -4 and -5 (Cryns and Yuan, 1998). Caspase-11 belongs to this caspase-1 subfamily of caspases (Wang et al., 1996). Since caspase-11 is essential for the activation of caspase-1 to produce mature cytokines like interleukin (IL)-1β, or IL-18, caspase-11 has been known to be a caspase that regulates inflammatory response (Wang et al., 1996; 1998). However, under certain pathological conditions caspase-11 can regulate apoptosis by directly activating caspase-3 (Kang et al., 2000; 2003). Therefore, caspase-11 is a dual regulator of apoptosis and inflammation.

These unique features of caspase-11, i.e., inducibility, wide range of expressing cells and dual regulation of both apoptosis and inflammation, can be used to develop an effective assay system to screen possible inducers or inhibitors of apoptosis/inflammation. In the present study, we generated a reporter construct where green fluorescence protein (GFP) expression is regulated by caspase-11 promoter. We observed dramatic induction of GFP in the cells transfected with this reporter construct after addition of known toxic chemicals. The fluorescence could be detected qualitatively using fluorescence microscope and qunatitatively using fluorometer. This result suggests that our reporter system can be used to screen pro- or anti-apoptotic/inflammatory molecules in a high-throughput manner.

Materials and Methods

Construction of caspase-11 promoter-GFP expression vector

Cloning of 1 kb caspase-11 promoter has been described by Schauvliege et al., (2004). Caspase-11 promoter fragment was cloned by PCR using *pfu* Turbo® (Stratagene) from pGL-caspase-11 promoter expression vector generously provided by Dr. R. Beyaert (Chent University, Belgium). The CMV promoter of the pEGFP vector was excised out using AseI and SmaI sites (pEGFPΔCMVp). The caspase-11 promoter fragment was then inserted into the pEGFPΔCMVp (Casp11p-GFP).

Cells and cell culture

Mouse neuroblastoma N2a cells were purchased from the ATCC (CCL-131) and maintained in DMEM (JBI, Korea) supplemented with 10% FBS (Invitrogen). Cells were subcultured every 2-3 days. Mouse embryonic fibroblasts (MEFs) were cultured from mouse embryos at day 13. Briefly, limbs, heads and tails were removed from the embryos and skin was carefully taken from the embryos. The removed skin was minced and digested with 0.05% Trypsin-EDTA solution for 15 min with occasional pipetting. The resulting single cell suspension was counted and seeded onto 100 mm culture dish in DMEM with 10% FBS. Astrocytes were cultured from 1 day-old mouse pups as described by McCarthy and de Vellies (1980) with minor modifications. For the fluorescence microscopy, cells were grown on poly-L-lysine-coated coverslips. For the spectrofluorometer reading, cells were grown on 96-wells. To measure the GFP fluorescence by specrofluorometry, SpectraMax Gemini (Molecular Device) was used (λ_{ex} = 400, λ_{em} = 508).

Transfection

Transfection was done using TransIT®-LT1 reagent (Mirus Bio Corp.) according to the manufacturer's protocol.

Immunoblot assay

For the detection of GFP and caspase-11, transfected cells were lysed in RIPA lysis buffer (50 mM Tris, 150 mM NaCl, 1% NP-40, 0.1% SDS) containing protease inhibitor cocktails (Boehringer Mannheim) and the lysates were centrifuged at 12,000 rpm for 10 min using benchtop microcentrifuge at 4°C. The supernatants were measured for the protein concentration using BCA assay kit (Pierce). The samples were separated on 12% SDS-PAGE and transferred to Immobilon PVDF membrane (Millipore). The membrane was blocked in blocking solution (Tris-buffered saline containing 0.1% Triton-X 100 (TBST) and 5% skim milk) for 1 hr at room temperature. The membrane was then incubated in the blocking solution containing appropriate amount of primary antibodies at 4°C overnight, followed by washing in TBST 3 times for 10 min each. The membranes were then incubated with secondary antibodies conjugated with horseradish peroxidase (Jackson Immuno Research). Following washing, the membranes were reacted with ECL reagent (Amersham) and then developed.

Antibodies and reagents

The antibodies for GFP and tubulin were purchased from Sigma and the antibody for caspase-11 was a generous gift from Dr. J. Yuan (Harvard Medical School, Boston).

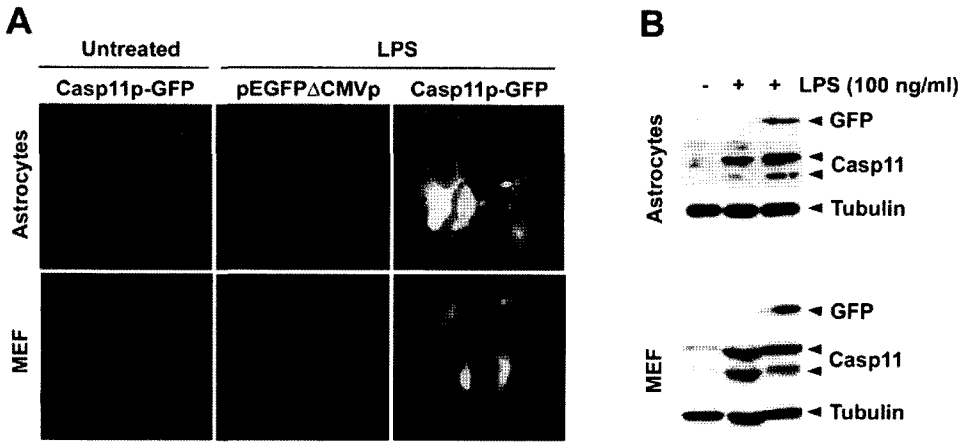

Fig. 1. Induction of GFP and caspase-11 in caspase-11 promoter-GFP-transfected cells. A, Mouse astrocytes and MEFs were transfected with caspase-11 promoter-GFP (Casp11p-GFP) or pEGFP vector without CMV promoter (pEGFPΔCMVp). After 12 hrs, the transfected cells were treated with LPS (100 ng/ml) and the expression of GFP was examined under a fluorescence microscope following 12 hr after the LPS treatment. B, The cells transfected with pEGFPΔCMVp (second lane) or Casp11p-GFP (first and third lanes) were treated with LPS for 12 hrs and the induction of GFP and caspase-11 was examined by immunoblot assay. Tubulin was probed for loading control.

Restriction enzymes were from NEB and plasmid purification kit was from Qiagen. All other reagents were purchased from Sigma chemicals otherwise stated. Mice (C57BL/6J) for the primary culture were purchased from Daehan BioLink.

Results and Discussion

To examine whether the caspase-11 promoter-GFP construct was properly operating, astrocytes and MEFs were transfected with the construct and examined for the induction of GFP following treatment of LPS, a potent inducer of caspase-11. As shown in Fig. 1A, astrocytes and MEFs transfected with the promoter-deleted vector (pEGFPΔCMVp) did not express GFP regardless of LPS treatment but cells transfected with caspase-11 promoter-GFP construct produced GFP only after the LPS treatment. Then we examined the induction of GFP and caspase-11 by immunoblot assay. As shown in Fig. 1B, expression of GFP was detected only in the LPS-stimulated cells transfected with the caspase-11 promoter-GFP construct in the LPS-stimulated cells. And endogenous caspase-11 was detected only in the LPS-treated cells as expected. These results indicate that the caspase-11-promoter-GFP was turned on in response to an inflammatory stimulus, LPS treatment, suggesting that this reporter system can be used to detect cell-damaging agents.

To further examine whether this reporter construct can respond to various toxic stimuli and the detection of the positive results can be made in a quantitative way, we plated the reporter-transfected cells in a 96-well plate and then measured the level of GFP expression by spectro-fluorometry following treatment of various toxic reagents. As shown in Fig. 2, an ER stress inducer like tunicamycin (Hu et al., 2004), oxidative stress inducers like ethanol and xanthine plus xanthine oxidase (Olney et al., 2003; Jaeschke, 2002) and LPS all increased GFP fluorescence in the reporter-transfected N2a neuroblastoma cells.

We have previously reported that the Scutellariae radix (SR) extracts that have anti-oxidant and anti-inflammatory activities (Gao et al., 1999) suppressed the induction of caspase-11 following ethanol treatment (Kang et al., 2005). Thus, we tested if the SR extract can suppress the GFP fluorescence of the reporter-transfected cells. Indeed, the SR extacts suppressed not only the basal level of fluorescence but also the fluorescence induced by ethanol treatment. Three known components of the SR extracts, wogonin, baicalin and baicalein (Gao et al., 1999; Shieh et al., 2000) all suppressed the basal level of the GFP-induced fluorescence. To confirm if this suppression of the fluorescence actually reflect the suppression of caspase-11 induction, we examined the level of caspase-11 after treatment of ethanol with or without SR extracts. As shown in Fig. 3, ethanol induced both GFP and caspase-11 protein in the cells transfected with caspase-11 promoter-GFP reporter construct. However, 30 min-pretreatment of the SR extracts suppressed the expression of both GFP and caspase-11 as we have previously reported.

This result suggests that our reporter construct can be used to screen anti-cytotoxic or anti-inflammatory compounds as well as cytotoxic or pro-inflammatory compounds. This implies that our reporter system has potential value as a tool to screen useful compounds that can be developed for therapeutics to treat various diseases accompanying cell death and/or inflammation. Furthermore, the level of toxic compounds that induced GFP fluorescence in Fig. 2 was much less than that is known to induce cell death,

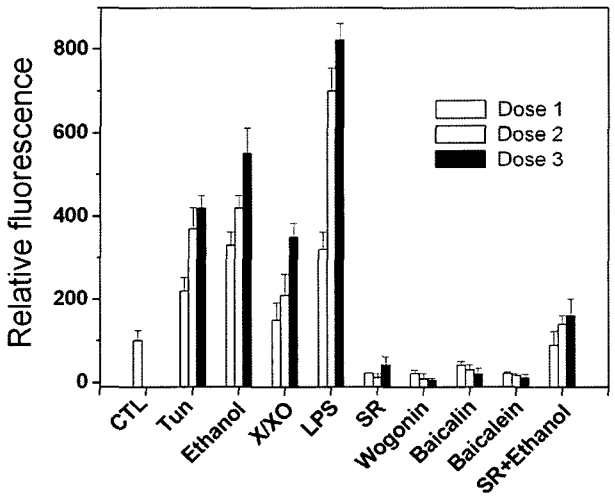

Fig. 2. Measurement of the intensity of GFP fluorescence following various drug treatment. N2a cells were transfected with Casp11p-GFP for 12 hrs and treated with the indicated agents for another 12 hrs. Three different doses were used for each agent (doses 1, 2, and 3). For tunicamycin, 10, 100, and 1000 ng/ml were chosen; ethanol, 2.5, 5, 10 mg/ml; Xanthine/Xanthine oxidase (X/XO), 1/1, 5/5, 10/10 mM/mUnit; LPS, 10, 100, 1000 ng/ml; SR extracts, 1, 5, 10 μg/ml; wogonin, baicalin, baicalein, 1, 5, 10 μg/ml. For the co-treatment of SR extracts and ethanol, SR extracts were 30 min-pretreated. CTL, untreated control. Shown are means ± S.D. (n = 3).

indicating our reporter system is very sensitive. Thus, the caspase-11 promoter-GFP reporter system can be used to detect pro-apoptotic or pro-inflammatory molecules as well.

Since activation of caspase-11 has been reported in many mouse models of diseases like brain ischemia, multiple sclerosis, Parkinson's disease, heart ischemia-reperfusion and septic shock (Kang et al., 2000; Hisahara et al., 2001; Furuya et al., 2003; Wang et al., 2005; Kang et al., 2002), specific inhibitors of caspase-11 will be of great therapeutic value. However, since caspase-11 is a murine caspase, identification of its huaman orthologue is necessary. Currently, caspase-5 is reported to be a human orthologue of caspase-11 (Lin et al., 2000). The expression of caspase-5 is upregulated by LPS like caspase-11 (Lin et al., 2000). In addition, caspase-5 regulates IL-1β maturation and can interact with caspase-1 (Martinon et al., 2002). The second possible human orthologue of caspase-11 is caspase-4 whose expression is induced by interferons (Ahn et al., 2002). Considering the involvement of caspase-11 under various pathological conditions, identification of true orthologue of caspase-11 in human is urgently required.

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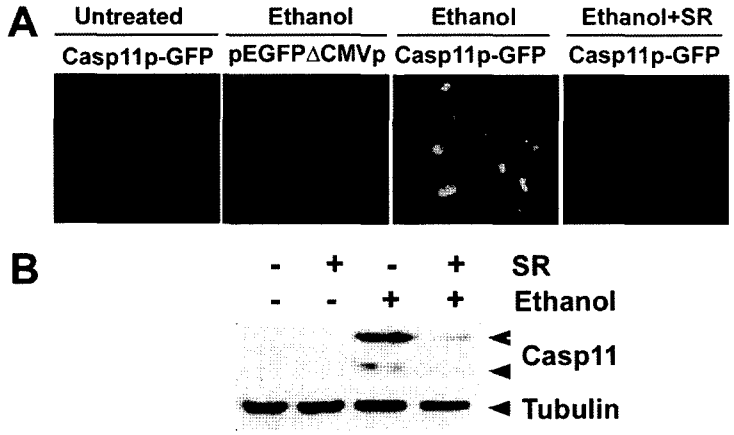

Fig. 3. Suppression of caspase-11 induction by the SR extracts in the cells treated with ethanol. A, To confirm the reduction in the GFP fluorescence in the Casp11p-GFP-transfected cells reflect the suppression of the caspase-11 expression, the cells transfected with Casp11p-GFP for 12 hrs were treated with SR or SR+ethanol for another 12 hrs. The expression of GFP was examined by fluorescence microscopy. Untreated cells transfected with Casp11p-GFP and LPS-treated cells transfected with pEGFPΔCMV did not express GFP. Note the pretreatment of the SR extracts suppressed the ethanol-induced GFP expression. B, The expression of caspase-11 in Casp11p-GFP-trnasfected cells as in panel A were examined by immunoblot assay using anti-caspase-11 antibody. Tubulin was probed for loading control.

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