Essential requirement of Apolipoprotein J (clusterin) signalling for IkB expression and regulation of NF-κB activity.

Giorgia Santilli 1,2, Bruce J. Aronow3 and Arturo Sala 1,2

1) Molecular Haematology and Cancer Biology Unit, Institute of Child Health, UCL, London WC1N, United Kingdom.
2) Dipartimento di Oncologia e Neuroscienze, Sez. Oncologia Medica, University G. D’Annunzio, 66100 Chieti, Italy
3) Division of Developmental Biology, Children’s Hospital Medical Center, Cincinnati, OH 45229

Correspondence: Arturo Sala, Molecular Haematology and Cancer Biology Unit, Institute of Child Health, UCL, London WC1N 1EH, United Kingdom.
Tel: 44-020 79052714
FAX: 44-020 7813-8100
a.sala@ich.ucl.ac.uk

running title: Apolipoprotein J is required for IkB stability.
Abbreviations: ApoJ, apolipoprotein J; TNF, tumour necrosis factor; IkB, inhibitor of KappaB; IKK, inhibitor of KappaB Kinase; DOXO, doxorubicin.

Abstract

ApolipoproteinJ/clusterin is an enigmatic protein highly regulated in inflammation, apoptosis and cancer. In spite of extensive studies, its biological function has remained obscure. Here we show that apolipoproteinJ inhibits neuroblastoma cell invasion. Since this function can be regulated by NF-kB, we explored the possibility that apolipoproteinJ might interfere with NF-kB signalling. Ectopic apolipoproteinJ expression strongly inhibited NF-kB activity in human neuroblastoma cells and murine embryonic fibroblasts by stabilising inhibitors of NF-kB (IkBs). Steady state levels of IkB proteins are drastically reduced in mouse embryo fibroblasts after disruption of the apolipoproteinJ gene. Absence of ApolipoproteinJ causes reduction of IkB stability, a TNF-dependent increase in NF-kB activity, increased transcription of the NF-kB target gene c-IAP and downmodulation of p53 protein. These results suggest that an unexpected physiological role of apolipoproteinJ is to inhibit NF-kB signalling through stabilisation of IkBs and that this activity may result in suppression of tumour cell motility.
ApolipoproteinJ/clusterin (ApoJ) is an ubiquitously expressed secreted protein whose presumed functions include cell-cell interactions, inhibition of complement-mediated cytolysis, and chaperone activity (1,2). The product of the ApoJ gene consists of a predominant form of about 75 kD, which is secreted in the extracellular spaces and body fluids. The 449-amino acid primary polypeptide chain of human ApoJ is proteolytically cleaved into the alpha and beta chains. Five disulfide bridges link the two subunits to obtain the mature form of the protein. ApoJ is highly conserved in different species, showing a 70-80% protein homology in mammals (1). Its expression is low in normal conditions but is greatly induced by oxidative, thermal and apoptotic stimuli, suggesting that ApoJ function could be directly or indirectly related to the cell suicide program and/or the stress response.

The biological role of ApoJ is still controversial. Several studies have suggested that ApoJ has antiapoptotic activity, protecting cultured cell lines against a variety of stress signals. Exogenously supplied ApoJ protects tumour cells from cytokine- or drug-induced apoptosis and inhibition of ApoJ results
in the increased sensitivity of cancer cells to chemotherapeutic drugs (3-6). However, a nuclear ApoJ form has been described which marks cells for apoptosis through its association with the DNA-repair protein KU70 (7).

The generation of knock-out mice have complicated the picture. Mouse development is not affected by the absence of ApoJ (8). However, ApoJ null mice show increased sensitivity to autoimmune myocarditis, suggesting a role for ApoJ in protecting the heart tissue from postinflammatory destruction (8). In contrast, in the absence of ApoJ, mice are partially protected after hypoxic injury suggesting that it can have a negative role in neuronal survival (9).

The prevailing hypothesis on the biological role of clusterin suggests that it is involved in the clearance of toxic substances from extracellular spaces through its ability to bind to unfolded proteins, cell debris or immune complexes. ApoJ binds to the endocytic receptor Megalin (LRP-2), and the clearance effect would be achieved by internalisation of the receptor-ligand complex and lysosomal degradation of the toxic substances (1,2). ApoJ has been shown to be highly regulated during tumour progression and to be a B-MYB oncoprotein-target gene (10,5). In this study we have investigated whether ApoJ expression may change tumour cell characteristics by impinging on specific signal transduction pathways.
Materials and methods

Cell cultures and in vitro invasion assay. The human neuroblastoma cell line LAN5 (11), the amphotropic-packaging cell line Phoenix A (ATCC SD3444) and early passage Mouse Embryonic Fibroblasts (MEFs) were maintained in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM glutamine, and 2 mM penicillin-streptomycin (GIBCO). For in vitro invasion assays 5x10^5 cells were resuspended in 1 ml medium containing no serum and seeded on top of the invasion assay chamber. Bottom chamber was filled with medium containing 5% FCS that served as chemo-attractant. After 24 hours, cells were collected
in the bottom chamber and scored as described in the manufacturer’s instructions (BD Biosciences). Retroviral infection of LAN5 and MEFs cells were carried out as described previously (12).

**Plasmids.** The complete Apolipoprotein J cDNA was amplified by RT-PCR from LAN5 cells. Correct amplification was assessed by sequencing. The blunt PCR product was cloned into Hpa-I digested MIGR1 retroviral vector (12). The ApoJ cDNA was cloned into Bam-HI, Eco-RV digested pCDNA3 to obtain pCDNA3-APOJ. The NF-kB luciferase reporter plasmid pNFKBLUC, containing 4 copies of the NF-kb consensus sequence, is from Clontech. A c-Jun-responsive reporter vector, containing AP1-binding sites from the c-Jun promoter cloned upstream the luciferase gene, was obtained from Dr. J. Ham. The mutant IkB super-repressor cDNA was obtained by mutagenesis of the RT-PCR amplified product, which was subcloned into the MIGR1 vector. Expression of the construct was verified by WB analysis with IkB alpha antibody and its inhibitory function was verified by its ability to suppress activity of the pNFKB-LUC plasmid in luciferase assays. The retroviral vector encoding for the constitutively active IKK kinase (IKKe) was published (13).

**Luciferase Assays.** MEFs and LAN5 cells were transfected with Lipofectamine 2000 (Invitrogen) with 0.5 µg of the pNFKB-LUC reporter plasmid in combination with 0.5 µg of empty pCDNA3 or pCDNA3-APOJ in 35-mm wells. After 18 hours, cells were exposed to 0.5µg/ml doxorubicin or 50ng/ml of tumour necrosis factor (TNF) for 8 hours, harvested and monitored for luciferase activity with a dual-luciferase assay kit, following the manufacturer’s instructions (Promega). Light emission was evaluated with the
aid of a luminometer and expressed as light units, which were normalised with a renilla luciferase reference plasmid.

**Reverse Transcription (RT)-PCR.**

After reverse transcription, PCR was performed with the following primers:

APOJ Forward 5’ ATGAAGATTCTCTGCTGCG 3’, APOJ reverse 5’
CTTCACCACCACCTCAGT

GAPDH forward 5’ ACCACAGTCCATGCCATCAC, GAPDH reverse 5’
TCCACCACCTCTGTTGCTGTA

For semi-quantitative RT-PCR, preliminary experiments were carried out to determine the linear range of amplification. c-IAP-1 primers were the following: forward primer 5’ GGACATTAGGAGTCTTCCC; reverse primer 5’ GCTGGGAAGTGACAGTGAAC.

**Protein stability and in vitro kinase assays**

For protein stability experiments, 293 cells were transfected with the plasmid encoding for the IκB mutant (ser 32/36) and incubated with cycloheximide (25µg/ml) for the indicated times with or without protease inhibitor. Protease inhibitors were dissolved in DMSO, which was added (0.2%) to untreated cells as a control. We have assessed IκB mutant expression by western blotting with a HA-tag antibody. The in vitro kinase assay was performed as described (14).

**Results**
ApoJ negatively regulates neuroblastoma cell invasion

We wanted to determine whether ectopic ApoJ expression interferes with the invasive behaviour of neuroblastoma cells. Since human neuroblastoma cells are difficult to transiently transfect, we subcloned the ApoJ cDNA into the MIGR1 retroviral vector and we transduced LAN5 cells with the ApoJ-expressing, or control, retroviruses. The MIGR1 vector contains the EGFP gene and allows simultaneous expression of the inserted cDNA through the presence of an IRES sequence (12). GFP-positive cells were isolated by FACS sorting, and subjected to in vitro invasion assays. ApoJ overexpression drastically reduced the number of cells migrating in the bottom of the well, suggesting that it may suppress cell invasion (Fig 1A). Cells transduced with a dominant-negative IκB (Inhibitor of KappaB)-alpha molecule, which behaves as a NF-κB super-repressor (15), were less prone to invade the matrigel substrate, suggesting that, as in other tumour models (16), NF-κB activity is important for neuroblastoma cell invasive properties (Fig 1B).

Consistent with this hypothesis, a constitutively active IKK kinase (IKKee), which induces degradation of inhibitors of KB (IκB) and activation of NF-κB, significantly promotes invasion of LAN5 cells in vitro (Fig 1C). Notably, concurrent ApoJ expression drastically reduced the number of IKKee-transfected cells migrating through the matrigel membrane, suggesting that ApoJ activity impinges on the NF-κB pathway (Fig 1C). To assess whether ApoJ could directly interfere with the IKK kinase, we carried out in vitro kinase assays, which established that ApoJ does not inhibit IKK kinase activity (fig 1D). The conclusion drawn from these experiment is (a) NF-κB is
required for in vitro invasion of neuroblastoma cells and (b) ApoJ inhibits this function downstream the IKK kinase.

**ApoJ specifically inhibits NF-kB activity and enhances stability of inhibitors of NF-kB in cancer cell lines.**

To directly assess whether ectopic ApoJ expression can modulate NF-kB activity, we transfected LAN-5 cells with a CMV-driven ApoJ cDNA together with a NF-kB-responsive promoter linked to luciferase. Basal or doxorubicin-induced NF-kB activity is strongly inhibited in LAN5 cells transfected with the ApoJ expression vector (Fig 2A). Similar results were obtained with another neuroblastoma cell line, SHSY5Y (not shown). The experiments described in the above section (Fig 1) insinuate that ApoJ biological effects could be achieved, at least in part, by interfering with factors downstream the IKK kinase. Indeed, we found that ApoJ expression enhances IkB-alpha/beta, but not tubulin, levels and that doxorubicin downmodulates IkB expression more efficiently in control-, than in ApoJ-, transduced cells (Fig 2B). Inhibitors of Kappa B (IkB) proteins play a key role in maintaining the NF-kB molecule in an inactive state (17,18).

Interestingly, extrinsic expression of ApoJ prolongs the half-life of the IkB ser 32/36 mutant (IkB-M, used in the experiment described in fig 1B), which is refractory to signal-dependent (i.e. IKK-dependent) degradation (Fig 2C). Both signal-dependent and independent IkB degradation can occur through IkB ubiquitination, which is followed by proteasome degradation (19). Accordingly, we found that the proteasome inhibitor MG132 and ApoJ, but not the calpain inhibitor calpeptin, prolongs ectopically expressed IkB-M half life.
(Fig. 2D). These results suggest that ApoJ modulates both signal-dependent and independent IkB turnover downstream the IKK kinase.

To assess specificity of the ApoJ suppressing effect on NF-kB activity, we transfected neuroblastoma cells with NFkB- or AP-1-responsive promoters, with or without the ApoJ expression vector, and carried out luciferase assays. ApoJ induced a ~70% inhibition of the NF-kB-responsive promoter, but it decreased only slightly Jun promoter activity, suggesting that ApoJ significantly suppresses NFkB-, but not AP-1-dependent transactivation (Fig 2 E,F). Deletion of the ApoJ amino-terminus, containing the protein export signal, did not change its activity, indicating that cytoplasmic, but not secreted, ApoJ is involved in suppression of NF-kB activity (data not shown).

ApoJ knockout causes enhanced NF-kB response after genotoxic stress in primary fibroblasts.

To explore the role of ApoJ signalling in a normal cell context, we assessed NF-kB activity in embryonic fibroblasts (MEFs) from normal or ApoJ knockout mice. Basal activity of the NF-kB reporter plasmid was higher in ApoJ knockout MEFs compared to control cells (3.6±0.65 vs 6.8±1.2, Fig 3). Notably, ApoJ null, but not control, MEFs show a peak of NF-kB activity after exposure to doxorubicin (Fig 3 A,B). Reconstitution of ApoJ expression results in suppression of the NF-kB response in doxorubicin-treated ApoJ null MEFs (Fig 3B). Expression of endogenous ApoJ in primary fibroblasts was detectable in control but not in ApoJ knockout MEFs, as expected (Fig 3C).

Reduced IkB protein levels in ApoJ knockout fibroblasts.
A classical signalling pathway that leads to NF-kB activation is that triggered by the tumour necrosis factor receptor. IkB-beta, and to a lesser extent IkB-alpha, protein levels are significantly reduced in fibroblasts from ApoJ knockout embryos in the presence of TNF stimulus (Fig. 4A). Interestingly, IkB beta protein expression is drastically decreased in ApoJ knockout MEFs, even in the absence of TNF (Fig. 4A). While protease inhibitors did not modulate IkB-beta levels in normal fibroblasts, the proteasome inhibitor MG132, but not the calpain inhibitor calpeptin, rescued IkB protein levels in ApoJ knockout cells (Fig. 4B). This experiment demonstrates that, even in the absence of specific stimuli, IkB-beta is more susceptible to proteasome-dependent degradation in the ApoJ knockout background, which probably justifies the higher basal NF-kB activity observed in ApoJ-null cells. Northern analysis shows that IkB-beta mRNA is robustly expressed in ApoJ knockout cells, indicating that endogenous ApoJ expression does not affect transcription of the IkB-beta gene (Fig 4C). Reconstitution of ApoJ expression restores, at least in part, IkB-beta protein levels in ApoJ knockout cells (Fig 4D, compare lanes 2 and 3), confirming that differences in IkB expression are related to the absence of ApoJ and not due to clonal variations. Overall these results indicate that endogenous levels of ApoJ are critically required for steady state IkB-beta, and to a less extent IkB-alpha, protein levels.

**NF-kB target genes expression is altered in ApoJ knockout cells.**

An important prediction drawn from these experiments is that NF-kB target genes expression should be modulated in the absence of ApoJ. We have verified this hypothesis by transiently transfecting ApoJ knockout, or control,
MEFs with the NF-κB reporter vector in the presence of TNF. TNF-dependent, as well as independent, NF-κB activity is substantially increased in ApoJ knockout cells, as compared to wild type fibroblasts (Fig 5A, bottom). We furthered this finding by examining mRNA levels of the NF-κB-target gene c-IAP (20). In comparison to wild type cells, c-IAP-1 mRNA levels were increased in both TNF-treated, or untreated, ApoJ knockout fibroblasts (Fig 5A, upper side). We have repeated this experiment with two more independent MEF preparations with similar results. It has been shown, by Verma and co-workers, that p53 protein stability is negatively regulated by endogenous levels of NF-κB and that p53 levels are decreased in mouse embryo fibroblasts with activated NF-κB (21). In agreement with this observation, we found that p53 protein levels are significantly decreased in ApoJ knockout fibroblasts (Fig. 5B). Albeit basal expression of p53 protein is reduced in ApoJ knockout fibroblasts, it is detectable after longer western blot exposures and it is induced by chemotherapeutic drugs treatment (data not shown). Overall these experiments demonstrate that the gene and protein expression patterns of apolipoprotein J knockout fibroblasts are consistent with that of cells with activated NF-κB.

Discussion

NF-κB is a transcription factor that plays a pivotal role in numerous cellular processes and is constitutively expressed in many cancer cell types. Its activity is regulated through interaction with inhibitory molecules (IκBs) that modulate NF-κB function by sequestering it in inactive cellular sites or by suppressing its transcriptional activity (17,18). IκBs, are, in turn, subjected to phosphorylation by kinases (IKKs) that mark for their destruction, or
inactivation, resulting in the release of active NF-kB (17,18). Modulation of NF-kB activity is thought to be important for cell survival, stress response, immunity, cell motility, proliferation and transformation (16,17,18,20).

In this investigation we have reported the crucial role of ApoJ in controlling IkB expression and NF-kB activity in normal and tumourigenic cells. Regulation of NF-kB activity could satisfactorily explain ApoJ opposing effects in different systems. Heart damage is exacerbated in ApoJ deficient mice subjected to experimental autoimmune myocarditis (8). ApoJ expression could be required to suppress excessive NF-kB activation, which determines inflammation and cardiomyocyte apoptosis in autoimmune myocarditis in the rat (22). This hypothesis is also suggested by our observation that disruption of the ApoJ gene results in increased NF-kB response evoked by the inflammatory cytokine TNF (Fig 5). In contrast, ApoJ contributes to brain injury in a study of experimental hypoxia-ischemia where it has been shown that exogenous clusterin increases cortical neuron death induced by oxygen/glucose deprivation (9). Since NF-kB activation is required for the survival of cortical neurons subjected to ischemia (23), suppression of NF-kB function could be at the base of clusterin neurotoxicity.

Loss of ApoJ expression in cells that depend on NF-kB activity for chemoresistance, proliferation or invasion could lead to tumour progression. In this regard, we show that acutely expressed ApoJ can drastically reduce the in vitro invasive properties of neuroblastoma cells (Fig 1). Consistent with our results, Sivamurthy and co-workers have recently shown that exogenously supplied ApoJ inhibited vascular smooth muscle cell migration in
microchemotaxis chambers (24). TNF-induced vascular smooth cell migration depends on NF-κB-mediated regulation of key cytokines (25). In preliminary experiments we have observed that ApoJ suppresses the metastatic phenotype of neuroblastoma xenografts in immunocompromised mice (Santilli et al, manuscript in preparation).

The ApoJ gene chromosomal location is at 8p21, a region commonly deleted in human cancers. We propose that ApoJ could be a tumour suppressor protein required to control tumourigenic signals emanating from the NF-κB pathway. This could explain why advanced testicular tumours show greatly reduced expression of ApoJ compared to lower grade tumours or normal tissue (26). In other cancer types, ApoJ expression could be advantageous through reduced expression of NF-κB-regulated death receptors and increased resistance to genotoxin-induced apoptosis, as previously reported (27). Deletion of the protein export signal does not change the ability of ApoJ to regulate NF-κB (data not shown), suggesting that this biological function depends on intracellular ApoJ. How ApoJ mechanistically regulates IkB protein turnover needs to be clarified. We have determined that ApoJ does not directly bind to IkB alpha or beta (data not shown). The stabilisation effect could be achieved by direct regulation of IkB-interacting proteins or relevant ubiquitin ligases. In this regard, a recently described new class of RAS-like proteins suppress NF-κB function by directly binding to IkB molecules, resulting in their stabilisation (14). Verification of these hypotheses will be the subject for future investigations.
References

1) Jones, S.E, Jomary, C. (2002) *Int. J. Biochem. Cell. Biol.* 34:427-431.

2) Wilson, M.R., Easterbrook-Smith, S.B. (2000) *Trends Biochem. Sci.* 25:95-98.

3) Miyake, H., Chi, K.N., Gleave, M.E. (2000) *Clin. Cancer. Res.* 6:1655-1663.

4) Sintich, S.M., Steinberg, J., Kozlowski, J.M., Lee, C., Pruden, S., Sayeed, S., Sensibar, J.A. (1999) *Prostate.* 39:87-93.

5) Cervellera, M., Raschella, G., Santilli, G., Tanno, B., Ventura, A., Mancini, C., Sevignani, C., Calabretta, B., Sala A. (2000) *J. Biol. Chem.* 275:21055-21060.

6) Sensibar, J.A., Sutkowski, D.M., Raffo, A., Buttyan, R., Griswold, M.D., Sylvester, S.R., Kozlowski, J.M., Lee, C. (1995) *Cancer Res.* 55:2431-2437.

7) Yang C.R., Leskov K., Hosley-Eberlein K., Criswell T., Pink J.J., Kinsella T.J., Boothman D.A. (2000) *Proc Natl Acad Sci U S A.* 97:5907-12.

8) McLaughlin, L., Zhu, G., Mistry, M., Ley-Ebert, C., Stuart, W.D., Florio, C.J., Groen, P.A., Witt, S.A., Kimball, T.R., Witte, D.P., Harmony, J.A., Aronow, B.J. (2000) *J. Clin. Invest.* 106:1105-1113.
9) Han, B.H., DeMattos, R.B., Dugan, L.L., Kim-Han, J.S., Brendza, R.P., Fryer, J.D., Kierson, M., Cirrito, J., Quick, K., Harmony, J.A., Aronow, B.J., Holtzman, D.M. (2001) *Nat. Med.* 7:338-343.

10) Trougakos I.P., Gonos E., S. (2002) *Int J Biochem Cell Biol.* 34:1430-48.

11) Seeger, R.C., Danon, Y.L., Rayner, S.A., Hoover, F. (1982) *J. Immunol.* 128:983-989.

12) Pear, W.S., Nolan, G.P., Scott, M.L., Baltimore, D. (1993) *Proc. Natl. Acad. Sci. U S A.* 90:8392-8396.

13) Huber M.A., Denk A., Peter R.U., Weber L., Kraut N., Wirth T. (2002) *J Biol Chem.* 277:1268-75.

14) Fenwick C., Na S.Y., Voll R.E., Zhong H., Im S.Y., Lee J.W., Ghosh S. (2000) *Science.* 287:869-73.

15) Didonato, J., Mercurio, F., Rosette C., Wu-Li J., Suyang H., Ghosh S., Karin M. (1996) *Mol. Cell. Biol.* 16:1295-1304.

16) Garg A., Aggarwal B.B. (2002) *Leukemia.* 16:1053-68.

17) Ghosh S., Karin M. (2002) *Cell. Suppl.* S81-96.

18) Karin M., Ben-Neriah Y. (2000) *Annu Rev Immunol.* 18:621-63.
19) Pando M.P., Verma I.M. (2000) *J Biol Chem.* 275:21278-86.

20) Wang C.Y., Mayo M.W., Korneluk R.G., Goeddel D.V., Baldwin A.S. Jr. (1998) *Science.* 281:1680-3.

21) Tergaonkar V., Pando M., Vafa O., Wahl G., Verma I. (2002) *Cancer Cell.* 1:493-503.

22) Yokoseki, O., Suzuki, J., Kitabayashi, H., Watanabe, N., Wada, Y., Aoki, M., Morishita, R., Kaneda, Y., Ogihara, T., Futamatsu, H., Kobayashi, Y., Isobe, M. (2001) *Circ. Res.* 89:899-906.

23) Blondeau N., Widmann C., Lazdunski M., Heurteaux C. (2001) *J Neurosci.* 21:4668-77.

24) Sivamurthy N., Stone D.H., Logerfo F.W., Quist W.C. (2001) *J Vasc Surg.* 34:716-23.

25) Wang Z, Castresana MR, Newman WH. (2001) *FEBS Lett.* 508:360-4.

26) Behrens P, Jeske W., Wernert N., Wellmann A. (2001) *Pathobiology* 69:19-23.

27) Spalding, A.C., Jotte, R.M., Scheinman, R.I., Geraci, M.W., Clarke, P., Tyler, K.L., Johnson, G.L. (2002) *Oncogene.* 21:260-271.
Acknowledgments

Giorgia Santilli is a recipient of a FIRC fellowship. This study was partially supported by an Institute of Child Health Pump-Prim ing Grant.

Alain Mauviel, Anastasis Stephanou, Warren Pear and Daniel Krappmann are gratefully acknowledged for plasmids.
Figures legends

Fig 1

ApoJ inhibits neuroblastoma cell invasion promoted by the NF-kB-activator IKK kinase.

LAN-5 cells were infected with a retroviral vector containing the ApoJ (panel A) or the NF-kB super-repressor IκB-M (panel B) cDNA. After FACS sorting, GFP-positive cells were subjected to in vitro invasion assay in invasion chambers as described in the material and method sections. (C), A LAN-5 cell line stably expressing exogenous ApoJ, or CMV-empty vector-containing cells, was infected with the IKK2ee retrovirus. After selection with
zeocyn, stable clones expressing ApoJ and IKK2ee were isolated and subjected, with appropriate control cell lines, to in vitro invasion assay. Histograms illustrate the means of quadruplicate wells. Error bars indicate standard deviations. These experiments were repeated three times and a typical experiment is shown. (D) LAN-5 cells were treated with 0.5µg/ml of doxorubicin for 2 hours. IKK kinase was immunoprecipitated with IKK antibody and used for in vitro kinase assays with GST-IkB-alpha fusion protein.

Fig 2
ApoJ suppresses NF-kB and stabilises IkB proteins in cancer cell lines.

A) We transfected LAN5 cells with the pNF-kB-Luc plasmid, with or without pCDNA3 vector containing the ApoJ cDNA. Cells were exposed to 0.5µg/ml of doxorubicin for 8 hours before luciferase assays. Error bars indicate standard deviations of normalised light units in triplicate wells. A typical experiment, out of several, is shown. B) GFP-positive LAN5 cells were FACS sorted from non-infected cells after incubation with retrovirus-containing supernatants. We monitored expression of relevant proteins by western blotting with the indicated antibodies before or after 8 hrs exposure to 0.5µg/ml of doxorubicin. Lanes 1 and 3, indicate empty vector MIGR1 infected cells. Lanes 2 and 4, indicate MIGR1-APOJ infected cells. C) 293 cells were concurrently transfected with empty- or ApoJ-containing CMV vector and the IkB-M vector. Stability of the IkB-M protein was verified by western blot analysis after cycloheximide (CHX) block for the indicated times. D) 293 cells were transfected as described in C. DMSO or protease inhibitors were added before exposing the cells to cycloheximide for 8 hours. IkB-alpha mutant and actin
levels were verified by western blotting. Densitometric reading of the proteins is indicated on top of the gels and is expressed as percent of IKB expression in the absence of treatments. E,F) Luciferase assays were performed as described in A.

Fig 3
Enhanced genotoxic stress-induced NF-kB response in ApoJ deficient fibroblasts.
We transfected control- (A) or ApoJ-deficient (B) MEF fibroblasts with the pNF-kB-Luc plasmid and the ApoJ vector, where indicated. Empty CMV vector was used to equalise the amount of DNA in the different transfections. Doxorubicin was added where indicated for 8 hours before luciferase assays. Error bars indicate standard deviations of normalised light units. A typical experiment is shown. C) Expression of ApoJ mRNA was monitored in control (+/+) or ApoJ-null (-/-) MEFs by RT-PCR with ApoJ-specific primers.

Fig 4
Endogenous ApoJ is critically required for IkB-beta protein stability in MEF fibroblasts.
A) Two independent primary fibroblast cell lines from wild type (indicated by the + sign) or ApoJ knockout (indicated by the – sign) mice were cultured without or with 50ng/ml of Tumour Necrosis Factor (TNF) for the indicated times. Cell lysates were subjected to western blot analysis with IkB -alpha and -beta antibodies. Equal loading of the lanes was assessed by stripping and reprobing with a beta actin antibody. B) wild type (+/+ or ApoJ knockout (-/-) MEFs were treated with the indicated protease inhibitors or 0.2% DMSO and harvested 8 hours later for western blot analysis with IkB-beta or actin
antibodies. C) Total cellular RNA was extracted from wild type or ApoJ knockout MEFs and subjected to northern blot analysis with a IkB-beta probe. Equal loading of the lanes was assessed by reprobing the blot with GAPDH. 20 µg of RNA were loaded per each lane. This experiment was repeated with other independent MEF lines with similar results. D) For reconstitution experiments, MEFs were infected with the control (lanes 1 and 2) or MIGR1-APOJ vector (lane 3). After cell sorting, GFP positive cells were subjected to western blot analysis with the indicated antibodies. Note that the ApoJ antibody detects only exogenous (human) ApoJ. This experiment was repeated twice.

Fig 5
TNF induces increased NF-kB response and transcription of c-IAP in ApoJ knockout MEFs.
A) (Upper panel) Semi quantitative PCR analysis was performed after mixing 1/10 of the reverse transcription products with the PCR reaction mixtures. Linear ranges of the reaction were determined empirically by analysing the PCR products after different cycle steps. Cycles numbers in the figure refer to amplification of c-IAP. GAPDH cycles were 20 and 25, respectively. Lanes 1: control MEFs; lanes 2: Control MEFs+ TNF; lanes 3: ApoJ knockout MEFs; lanes 4: ApoJ knockout MEFs + TNF. (Bottom panel histogram) MEFs from control (+/+) or ApoJ knockout (-/-) mice were transiently transfected with the NF-kB luciferase reporter plasmid as described in fig 2. The next day, cells were treated with 50ng/ml of TNF and harvested after 8 hours for luciferase assays. Error bars indicate standard deviations. This experiment was repeated twice with independent MEF preparations. B) p53 levels were detected by
western blot analysis of exponentially growing wild type (+/+ or ApoJ knockout (-/-) MEFs. Similar results were obtained with other MEFs preparations.
A

25 cycles

30 cycles

c-IAP
GAPDH

1 2 3 4
1 2 3 4

B

+/+  -/-

p53
actin
Essential requirement of Apolipoprotein J (clusterin) signalling for IKB expression and regulation of NF-kB activity
Giorgia Santilli, Bruce J Aronow and Arturo Sala

J. Biol. Chem. published online July 25, 2003

Access the most updated version of this article at doi: 10.1074/jbc.C300252200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts