14

Microenvironment/apoptosis

14-01/P

APOPTOSIS INDUCED BY THE 2-5A SYSTEM: MECHANISMS INVOLVED AND ROLE OF MITOCHONDRIA

Domingo-Gil E, Esteban M

Department of Molecular and Cellular Biology, Centro Nacional de Biotecnología, CSIC, Ciudad Universitaria de Cantoblanco, Madrid, Spain

The interferons (IFNs) are cytokines released from animal cells in response to a variety of stimuli including viral infections. IFNs exert a pleiotropic effect on cellular proliferation and host defence through the activity of a number of IFN-inducible genes. The best characterized IFN-induced proteins are 68-KDa protein kinase (PKR), the 2-5A system and the Mx family. The 2-5A system is composed by the 2´ 5´oligoadenylate synthetase 1 (2-5OAS-1) and 2-5A-dependent RNase (RNaseL), enzymes that play a key role in antiviral defence mechanisms. Activation of the 2-5A system by double stranded RNA (dsRNA) induces degradation of ribosomal RNAs, which results in a general inhibition of protein synthesis and apoptosis in mammalian cells. To obtain further information into the molecular mechanisms by which RNaseL induces apoptosis, we expressed human RNaseL and 2-5OAS in HeLa cells using recombinant vaccinia viruses and we analysed in detail different biochemical markers of apoptosis. In this expression virus-cell system, RNaseL induces apoptosis in a caspase-dependent manner (caspase 8, 9 and 2), and the RNase activity of RNaseL seems to be an upstream event for apoptosis induction. By subcellular fractionation assays, we found that both enzymes RNaseL and 2-5OAS, localize in the mitochondrial fraction of cells. The 2-5A system induces the release of cytochrome c from mitochondria to cytosol in a caspase dependent manner. The onset of apoptosis elicits the disruption of mitochondrial membrane potential ($\Delta\Psi m$), as well as the generation of reactive oxygen species (ROS). Moreover, the activation of RNaseL induces morphological alterations in the mitochondria. Apoptosis induced by the 2-5A system involves mitochondrial proteins, such as the human anti-apoptotic protein Bcl-2, which blocks both the apoptosis and the change of $\Delta\Psi m$ induced by the activation of RNaseL. These findings provide new insights into the molecular mechanisms of apoptosis induction by RNaseL, demonstrating the important role played by mitochondria in this process.

14-03/P

SSRNA VIRUSES INACTIVATE P53 AND INDUCE *NOXA*-DEPENDENT APOPTOSIS *VIA* POST-TRANSLATIONAL MODIFICATIONS OF IRF-1/3 & CREB

Lallemand C[1], Blanchard B[1], Lebon P[2], Tovey M.G[1]

[1]Laboratory of Viral Oncology, UPR CNRS 9045, Institut André Lwoff, Villejuif, France; [2]Laboratory of Virology, Université René Descartes, Hopital Cohin-Saint Vincent de Paul, Paris, France

In order to characterize the mechanisms underlying apoptosis induced by viral infection transcriptional activation of genes encoding members of the "BH3 only" family of proteins was analyzed during the course of virus infection. Among these genes only *NOXA* is transcriptionally activated by Vesicular Stomatitis virus (VSV), Sendai virus (SV), Measles virus, Herpes Simplex virus (HSV), or synthetic dsRNA, and at least in the case of VSV infection, is required for efficient virus-induced apoptosis of cells. Transcriptional activation of *NOXA* by VSV or SV is independent of p53 but requires the presence of IRF-1, IRF-3 and CREB. Binding to and transactivation of the *NOXA* promoter by each of these transcription factors is governed by post-translational modification involving different pathways for each factor. Thus, SV infection activates IRF-3 and CREB by phosphorylation triggered by TLR3 signalling, and a pathway involving calcium-independent phopholipase A2, respectively. In addition transactivation induced by IRF-1 during viral infection, correlates with a 10 kDa increase in its MW, suggesting a covalent linkage with a previously unknown regulatory polypeptide.

14-04/P

FOCAL ADHESION KINASE DETERMINES THE PATHWAY LEADING TO DEATH OR SURVIVAL IN TNFα-STIMULATED FIBROBLASTS

Sonoda Y, Takahashi R, Ichikawa D, Yoshida N, Aizu-Yokota E, Kasahara T

Department of Biochemistry, Kyoritsu University of Pharmacy, Shibakoen, Minato-ku, Tokyo, Japan

TNFα is a pleiotropic cytokine that elicits a wide range of cellular effects, including proliferation, differentiation, and apoptosis. However, what determines the pathways leading to death or survival remains to be unelucidated. In this study, we found that FAK-/- cells are more sensitive to TNFα-induced apoptosis in the presence of actinomycin D (ActD) and failed in the activation of NF-κB compared to FAK+/- cells. Prosurvival pathways are activated by the rapid recruitment of complex I, comprising TNFR1, TRADD, RIP and TRAF2, which leads to the activation of the NF-κB pathway. On the other hand, proapoptotic pathways are activated by complex II, the death-inducing signaling complex (DISC), which contains TNFR1, TRADD, RIP, and FADD, and procaspase-8 proteins. As TNFR1, TRADD, and RIP are included both in Complex I and DISC, we speculated that RIP might be a key protein. Accordingly, we examined whether RIP is included in compound I or DISC. Coimmunoprecipitation assays revealed that RIP associated with TRAF2 and FAK upon stimulation with TNFα in the presence of ActD in FAK+/- cells, but not in FAK-/- cells. Namely, RIP is included in complex I in FAK+/- cells, but not in FAK-/- cells. These events lead to the activation of NF-κB and the resistance to TNFα-induced apoptosis. On the other hand, on immunoprecipitation with procaspase-8, RIP and FADD were detected in FAK-/- cells, but not in FAK+/- cells. This shows that RIP is included in DISC in FAK-/- cells, but not in FAK+/- cells. Thus, the diverging point whether apoptosis occurs or not is the step of the formation of complex I or DISC. FAK might act as a scaffold protein in the formations of complex I and the activation of NF-κB. In conclusion, we first demonstrated that FAK determines the pathway leading to death or survival in TNFα/ActD-stimulated fibroblasts.

14-05/P

EXPRESSION OF THE HUMAN IL18 BINDING PROTEIN IN E. COLI CELLS AND STUDY OF ITS BIOLOGICAL PROPERTIES.

Yakushenko EV[1], Lopatnikova JA[1], Sennikov SV[1], Khrapov EA[2], Voronina EN[2], Filipenko ML[2], Kozlova JN[3], Pustoshilova NM[3], Kozlov VA[1]

[1] Institute of Clinical Immunology, Novosibirsk, Russia; [2] Institute of Chemical Biology and Fundamental Medicine, Novosibirsk, Russia; [3] "Center of an engineering immunology". Novosibirsk, Russia.

The aim of the present research was preparation of recombinant IL18BP and investigation of its biological activity. Human IL18BP was obtained by E. coli expression of a gene encoding mature IL18BP protein. Purified proteins were analyzed by SDS-polyacrylamide gel electrophoresis. By a method of Dot-analysis it is shown, that IL18BP is capable of binding IL18. The ability of IL18BP to bind with anti-IL18BP antibodies was determined by an electrochemiluminescence assay using polyclonal IL18BP antibodies. It was determined that prepared protein binds with anti-IL18BP antibodies. The biological activity of IL18BP was determined on ability to abolish the effects of IL18. The cultivating PBMC with ConA (5mkg/ml), ConA + IL18 and IL18 (40ng/ml) in the presence of IL18BP (80mkg/ml) led to statistically significant decrease of production of TNFα (75.5%, 94.6% and 75.8% accordingly). Thus, obtained recombinant IL18BP has characteristics of its biological activity, in particular cancels stimulating influence of IL18 to production of TNFα by PBMC *in vitro*. To test whether IL18BP neutralizes IL18 *in vivo*, we studied the effect of IL18BP on LPS-induced lethality in mice. Administration of IL18BP (5 mg/kg) abolishes lethality induced by a dose of LPS (10 mg/kg) equivalent to LD_{70} ($p \leq 0.00141$, log-rank test). Antimetastatic activity of IL18BP was studied on model of a hematogenous metastasis of cells of a melanoma B16. It is shown, that the introduction of IL18BP (s.c. or i.v.) results in statistically significant reduction of an integrative quantitative parameter (quantity of metastasises × the size of a metastasis) of tumoral process in lung of mice.

14-06/P

EPSTEIN-BARR VIRUS LATENT MEMBRANE PROTEIN 1 ENHANCES MATRIX METALLOPROTEINASE 3 INDUCTION IN HUMAN FIBROBLAST

Lee DCW[1], Chua DTT[2], Sham JST[2], Lau ASY[1]

[1]Immunology Research Laboratory, Department of Paediatrics and Adolescent Medicine, The University of Hong Kong, Pokfulam, Hong Kong Special Administrative Region, PR China; [2]Department of Oncology, The University of Hong Kong, Pokfulam, Hong Kong Special Administrative Region, PR China

Epstein-Barr Virus (EBV) latent infection is associated with human malignancies including Burkitt's lymphoma, gastric carcinoma and the highly invasive nasopharyngeal carcinoma (NPC). The oncogenic EBV latent membrane protein-1, LMP1, induces cytokines and cellular proteins including IL-8, vascular endothelial growth factor and matrix metalloproteinases (MMP-1, 2 and 9). MMPs are endopeptidases involved in the degradation of extracellular matrix proteins and their upregulation in cancer implicates their role in tumor metastasis. For example, MMP-3 induced by cytokines and cellular factors in cancer and non-cancerous stromal cells may result in the deregulation of E-cadherin, thus promoting tumor invasion. We hypothesize that EBV-LMP1 induces MMP expression to promote the progression of NPC. To delineate the oncogenic role of LMP1 in NPC, we first investigated the transcription of MMP-1, -2, -3 and -9 in NPC biopsies (n = 15) by Quantitative RT-PCR. Our results showed upregulation of MMP-1 and MMP-3 in the LMP1-positive biopsies, compared with the adjacent non-malignant nasopharyngeal tissues. We then cloned the LMP1 DNA from the NPC and transiently expressed it in MRC5 (human fibroblast). Following transfection, the induction kinetics of MMP-3 was analyzed by Q-PCR. The levels of MMP-3 mRNA increased significantly from 24h and reached its maximum at 32h, compared to the mock-transfected MRC5 cells. We also detected a significant increased expression of MMP3 protein in the LMP1-expressing cells at 48h post-transfection. Following the LMP1 protein expression in the transfectants, the LMP1-induced MMP-3 transcription was not blocked by subsequent addition of cycloheximide, indicating that new protein synthesis was not required for the transcriptional activation of MMP3. Taken together, we observed that MMP3 is upregulated in LMP1-positive NPC biopsies and LMP1-expression in fibroblasts is associated with MMP3 and cytokine expression. Our results suggest that LMP1 may contribute to invasiveness of NPC cells *via* the expression of MMP3 in fibroblasts. (Supported by Clinical Oncology Research Fund, Department of Clinical Oncology and Department of Paediatrics, HKU).

14-07/O

DEVELOPMENT OF TYPE I INTERFERON RESISTANCE IMPLICATES STAT2 AS A CRITICAL MEDIATOR IN THE INDUCTION OF APOPTOSIS

Gamero AM, Scarzello A, Romero AL

Laboratory of Experimental Immunology, National Cancer Institute, Frederick Maryland, United States

Type I interferons (IFN-α/β) are cytokines characterized for their antiviral, antiproliferative, and immunomodulatory activities. Although the antitumor effects of type I IFNs are well-known, the underlying mechanism that programs certain tumor cell lines to die with IFNα/β has remained elusive. To begin elucidating the mechanism of type I IFN-induced apoptosis, we induced IFN-α resistance in H123 cells, a variant of the human leukemic T-cell line Jurkat that is susceptible to the apoptotic effects of type I IFNs. H123 cells were subjected to chronic IFN-α stimulation. This strategy generated an IFN-α apoptosis resistant clone that lost expression of STAT2 during the selection process. Interestingly, the STAT2 deficiency in the IFN-α apoptosis

resistant H123 clone did not affect IFN-α stimulated activation of STAT1 and up-regulation of MHC class I molecules. Yet in response to IFNα, our STAT2 deficient H123 cells displayed defective cytochrome c release from mitochondria, loss of mitochondrial membrane depolarization, and impaired caspase-3 activation and ISGF3-mediated gene expression. Re-expression of STAT2 restored the phenotype of parental H123 cells. Notably, the level of re-expressed STAT2 was critical as a certain amount of STAT2 protein was required to induce apoptosis at levels similar to those detected in parental H123 cells. Thus our results strongly indicate that in addition to STAT1, STAT2 is also a critical signaling component in type I IFN-induced apoptosis. Our findings have clinical implications as the beneficial antitumor effects of type I IFNs may be compromised with development of IFN resistance due to a loss of STAT2 expression.

14-08/O

ALTERNATIVE SPLICING AND ALTERNATIVE INITIATION OF TRANSLATION LEAD TO MULTIPLE ISOFORMS OF CASPASE-5 IN HUMAN LYMPHOCYTES

Eckhart L[1], Kittel C[1], Gruber F[1], Gawlas S[1], Mildner M[1], Jilma B[2], Tschachler E[1]

[1]Department of Dermatology, Medical University of Vienna, Vienna, Austria; [2]Department of Clinical Pharmacology, Medical University of Vienna, Vienna, Austria

Caspase-5 is implicated in apoptosis and in the inflammatory response to lipopolysaccharide (LPS). Here we investigated the structure of the caspase-5 gene and the regulation of its expression in lymphocytes. We identified a novel exon 1 of the caspase-5 gene which was flanked by a region homologous to the promoter of caspase-11, i.e. the murine ortholog of human caspase-5. Like caspase-11 in the mouse, expression of caspase-5 was strongly upregulated by LPS in peripheral blood lymphocytes of healthy human volunteers who had received a bolus of 2 ng/kg LPS i.v. Lymphocytes expressed six alternatively spliced caspase-5 mRNA variants that encoded distinct protein isoforms. Overexpression studies and Western blot analysis revealed that the translation of the caspase-5 variants was initiated predominantly at a site within the novel exon 1 which generated a previously unknown N-terminus at the protein level. In addition, truncated caspase-5 protein variants were formed by utilization of alternative start sites of translation in exon 3. The isoforms of caspase-5 differed significantly in their propensity to induce apoptosis in 293 cells. Caspase-5/b, the structural equivalent of caspase-11, was most efficient in the activation of the apoptotic executioner protease caspase-3. In conclusion, our findings lead to a revision of the primary structure of the human caspase-5 gene and demonstrate that expression of caspase-5 is controlled by transcriptional and post-transcriptional mechanisms.

14-09/P

TUMOR SUPPRESSIVE PROTEIN GRIM-19 ASSOCIATES WITH SERINE PROTEASE HTRA2 FOR PROMOTING CELL DEATH

Sudhakar Kalakonda[1], Xinrong Ma[1], Srinivasa M. Srinivasula[2], Sekhar P. Reddy[3], Dhan V. Kalvakolanu[1]

[1]Greenebaum Cancer Center, University of Maryland School of Medicine, Baltimore, MD; [2]Laboratory of Immune Cell Biology, National Cancer Institute, Bethesda, MD; [3]Department of Environmental Health Sciences, The Johns Hopkins University Bloomberg School of Public Health, Baltimore, MD, USA

The interferon and retinoid combinations are used for the therapy of several human cancers. We and others have shown that this combination induces apoptosis for suppressing tumor growth. Using a genetic technique, we have described the isolation of a novel cell death regulatory protein GRIM-19. To further understand its mechanism of action, we have employed a yeast-two-hybrid screen and searched for its cellular partners. These studies identified HtrA2, a serine protease implicated in cell death control. HtrA proteases were originally implicated in mediating cellular stress-responses in bacteria. Some of the human homologues of HtrA proteins are also upregulated during ischemic damage. Here we show that GRIM-19 physically interacts with HtrA2 and augments cell death in an IFN/RA dependent manner. Catalytically active HtrA2 was required for promoting a synergistic cell death response induced by IFN/RA. In the presence of GRIM-19, the HtrA2 driven destruction of the anti-apoptotic protein XIAP is augmented. Furthermore, these interactions were disrupted by a HHV-8 coded oncoprotein, vIRF1, which conferred resistance to IFN/RA-induced cell death. This report shows a critical role for HtrA2 in a cytokine-induced cell death response for the first time and its inactivation by a viral protein.

14-10/P

CASPASE ACTIVATION DURING LYTIC INFECTION BY VACCINIA VIRUS

Melkova Z., Kalbacova M, Spisakova M, Liskova J

Institute of Imunology and Microbiology, 1st Medical Faculty, Charles University, Prague, Czech Republic

Vaccinia virus (VV), an orthopoxvirus successfully used during smallpox eradication campaign, remains widely used as a strong expression vector for various recombinant genes. Similarly to other poxviruses it encodes many homologues of cellular genes, including homologues of cytokines and their receptors, that manipulate host responses to virus infection. VV is generally considered to cause lytic infection, but it causes apoptosis of certain immune cells as well. In vivo, consequences of necrotic and apoptotic cell deaths differ sharply. Specifically, the type of cell death determines the nature of immune responses raised. Despite a well known immunogenicity of VV that induces very strong and long lasting immune responses, the mechanism of this induction is still not sufficiently explained. Accordingly, it is important to characterize activation of cell death pathways during VV infection. In our work, we demonstrate that VV-infected epithelial cells (BSC 40 and HeLa G) revealed features of necrosis, while caspase activation, typical feature of apoptosis, could be observed as well. We assessed caspase activation at three different steps of the apoptotic cascade using three different flow-cytometric methods (caspase activation by Caspa Tag™ Fluorescein Caspase (VAD) Assay Kit, Intergen Company; caspase activity by cleavage of Rhodamine 110, bis-(L-aspartic acid amide), Molecular Probes; cleavage of a death substrate cytokeratin 18 by M30 CytoDEATH, Fluorescein, Roche), observing higher caspase activity in VV-infected cells compared to mock-infected ones. Next, we characterized levels of caspase substrates by western blot analysis. We detected a marked decrease of cytokeratin 18 levels in VV-infected cells. However, we have not observed any cleavage of PARP, a typical caspase-3 and -7 substrate. Thus, VV infection of epithelial cells reveals signs of both apoptosis and necrosis, with depletion of ATP being a possible cause of the resulting necrosis. The nature of caspases or other proteases involved is currently being characterized.

14-11/O

OBSTRUCTIVE NEPHROPATHY: THE ROLE OF TOLL-LIKE RECEPTOR-2

Leemans JC[1], Teske GJD[1], Stokman G[1], Claessen N[1], van der Poll T[2], Weening JJ[1], Florquin S[1]

[1]Department of Pathology; [2]Experimental Internal Medicine, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands.

Introduction: Toll-like receptors (TLR) are highly conserved pattern recognition receptors that detect pathogen motifs and host material released during injury. Renal TLR2 mRNA is mainly expressed by tubular epithelial cells (TECs). Obstruction of the upper urinary tract has deleterious effects on TECs and is an important cause of renal insufficiency. The changes that occur in upper urinary tract obstruction include inflammation, tubular apoptosis and fibrosis that culminate in loss of renal mass and renal dysfunction. Since we showed that TLR2 is a key initiator of inflammation, tubular apoptosis and renal dysfunction in renal ischemia-reperfusion injury, we hypothesized that TLR2 might play a role in obstructive nephropathy. *Methods*: Wt and TLR2KO mice (n = 6/group) were subjected to unilateral ureteral

obstruction (UUO). Animals were sacrificed at 3, 7, and 14 days for determination of interstitial leukocyte influx (immunohistochemistry), TEC apoptosis (immunohistochemistry), local TGFbeta/HGF levels (ELISA), collagen deposition (picro-sirius red) and renal injury (PAS-D). Statistics were done by Mann-Whitney test. *Results*: We found that TLR2 plays a proinflammatory role after UUO injury, as reflected by significantly reduced amounts of interstitial neutrophils in kidneys of TLR2KO mice compared to Wt mice at all time points. TLR2 deficiency markedly attenuated TEC apoptosis and myofibroblast influx at respectively 7 and 14d. The amount of macrophages in the cortex were however similar in both groups (t = 3, 7, 14). TLR2 deficiency reduced the amount of renal active TGFβ levels while HGF levels were unaltered. TLR2 deficiency did not influence fibrosis as measured by renal collagen deposition. TLR2KO mice were not protected from UUO injury as reflected by similar renal histopathological injury scores when compared to Wt. *Conclusion*: Together, we showed that Toll-like receptor-2 mediates renal cell death, neutrophil, and myofibroblast influx but does not play a role in the development of fibrosis and renal injury in experimental obstructive nephropathy.

14-12/P

POXVIRUS AND THE CELL DEATH PATHWAY: VACCINIA VIRUS DOUBLE-STRANDED RNA BINDING PROTEIN E3L BINDS CENTRAL DEATH PATHWAY PROTEASE CASPASE-3

Farlow JE, Langland JO, Jacobs BL

Center for Infection Disease and Vaccinology, The Biodesign Institute, Arizona State University, Tempe, Arizona, USA 85287-5401

Programmed cell death or apoptosis is a complex and highly regulated process of cellular execution involved in development, inflammation, cell turnover, and defense against viral and bacterial pathogens. Apoptosis is mediated by a family of cysteine proteases called caspases. Ligands initiating the extrinsic apoptotic signaling pathway including tumor necrosis factor alpha, FasL, and interferon which result in activation of downstream effector caspases. Activation of the intrinsic apoptotic pathway results in cytochrome c release from mitochondria, formation of apoptosomes, and activation of effector caspases. Both arms of the apoptotic signaling cascade converge upon and activate the central effector caspase, caspase-3. Caspase-3 cleaves downstream cell death substrates in both the cytoplasm and nucleus resulting in chromatin condensation, DNA fragmentation, and, ultimately, cell death. Vaccinia virus (VV) employs unique strategies to counter infection-induced apoptosis. While VV encodes serine-proteinase inhibitors (SPI), two of which (SPI-1,2) function as anti-apoptotic proteins, we report here that the VV dsRNA-binding protein E3L binds the central death pathway protease Caspase-3. Furthermore, microarray analysis of apoptosis-related transcripts revealed distinct variations in gene expression patterns following wild-type VV infection. Caspase-3 mRNA transcripts in wild-type E3L infected cells were up-regulated 16-fold over E3L deletion mutants. Caspase-8, Caspase-10 (FLICE2), and caspase recruitment domain 14 (CARD14) mRNA transcripts were also upregulated. Our preliminary results indicate the VV E3L protein may inhibit an array of host apoptosis-related proteins in order to counter the cell death pathway induced in poxvirus infection.

14-13/O

REGULATION OF IMMUNE HOMEOSTASIS BY A NOVEL CYTOKINE-INDUCIBLE GENE

Fujii H, Hoshino A

New York University School of Medicine, New York, NY, USA

Cytokines control cell differentiation, proliferation, and function by regulating gene expression program. Elucidation of the functional roles of cytokine target genes is essential for understanding mechanism of cytokine action. However, target genes of growth-promoting cytokines are largely unknown. We identified a novel cytokine-inducible gene, *cyclon (cytokine-induced protein with coiled-coil domain)*, by DNA microarray analysis. This gene is induced in receptor-reconstituted Ba/F3 cells by various growth-promoting cytokines including interleukin (IL)-3, IL-2, and erythropoietin. In addition, *cyclon* mRNA expression is induced in both CD4 (+) and CD8 (+) peripheral T cells by anti-CD3 Ab stimulation. Mouse and human *cyclon* genes encode proteins with 426 and 360 amino acid residues, respectively. Cyclon protein consists of amino-terminal repetitive sequences consisting of serine and proline residues and a carboxyl-terminal coiled-coil domain. Immunofluorescent and biochemical studies showed that Cyclon protein resides in the nucleus. Cyclon is a phosphorylated protein. Promoter analysis using a novel reporter system revealed that *cyclon* gene induction by IL-3 is mediated by redundant promoter elements between -185 and -49 bp upstream of the initiation methionine. These elements contain conserved binding sites of CREB/ATF, c-Ets-1, MZF-1, and an element that does not contain binding sites of any known DNA-binding proteins. Transgenic expression of Cyclon using hCD2 cassette in common cytokine receptor γ-chain (γc)-deficient mice markedly suppressed age-associated enlargement of spleen and reduced the number of activated T cells. These results suggest that Cyclon plays an important role in the regulation of immune homeostasis and suppression of activated T cells. Possible role of Cyclon in suppression of autoimmunity will also be discussed.

14-14/O

REVERSAL OF EPIGENETIC SILENCING TO ENHANCE INTERFERON STIMULATED GENES AND ANTITUMOR EFFECTS

Borden EC, Reu FJ, Bae SI, Jacobs B, Lindner D

Taussig Cancer Center, Cleveland Clinic Foundation, Cleveland, OH, USA

Postulating that silencing of interferon-stimulated genes (ISGs) by methylation of 5' CpG islands mediates resistance to IFNs, we assessed the DNA demethylating agent 5-AZA-deoxycytidine (5-AZA-dC). Treatment of human ACHN renal cell carcinoma (RCC) and A375 melanoma cells with either 5-AZA-dC or an antisense to DNA methyltransferase 1 (DNMT1) overcame complete resistance to apoptosis induction of IFN-α2 and IFN-β with up to 85% apoptotic cells resulting from the combinations. Western blot confirmed significant decrease in DNMT1 with 5-AZA-dC. No similar potentiation occurred in normal kidney epithelial cells. Other cells, such as SK-MEL28 nor SK-MEL3 that were resistant to apoptosis on TUNEL assay (<2% apoptotic cells) in response to even high doses of IFN-ß or IFN-α2, were also sensitive to the combination: >75% apoptotis (100 U/ml of IFN-β) without significant apoptosis from 5-AZA-dC alone (confirmed by caspase 3 and PARP cleavage). Genes essential for IFN-induced apoptosis and potentially silenced by DNA methylation, Stat1, Stat2, TRAIL, IRF1, TRAIL R1, TRAIL R2, XAF-1, were assessed by qPCR: in A375 cells 5-AZA-dC expressed XAF-1 about 70x while other genes were only minimally increased. In SK-MEL28 TRAIL R1 was increased in by 5-AZA-dC (>25x). siRNA to XAF1 inhibited IFN-induced apoptosis; conversely, overexpression of XAF1 overcame resistance to apoptosis by IFN-β. As occurred with apoptosis resistant melanoma cells *in vitro*, tumor growth inhibition of A375 melanoma xenografts resulted from treatment with 5-AZA-dC in combination with IFN-β, an effect not resulting from either single agent. Potentiated increase in apoptosis from IFNs with 5-AZA-dC in SK MEL 28 cells likely resulted from both an increase in TRAIL and in TRAIL R1 and in A375 cells from an increase in the pro-apoptotic XAF1. Data suggest that reactivation of silenced pro-apoptotic genes by DNA demethylators may restore IFN antitumor responses and could have clinical relevance.

14-15/P

CROSS-RESISTANCE TO INTERFERON INDUCED APOPTOSIS IN FAS LIGAND RESISTANT VARIANTS OF THE U937 CELL LINE

Blomberg J, Ruuth K, Lundgren E

Umeå University, Umeå, Sweden

Activation of the death receptor Fas (Apo-1/CD95) results in recruitment of several intracellular proteins into a death inducing signaling complex. In this complex the adaptor protein FADD interacts with the cytoplasmic death domain of the receptor and recruits initiator procas-

pases 8 and 10. Upon aggregation the caspases are autocatalytically cleaved and the active proteins initiate a signaling cascade leading to apoptosis. Interferon induced apoptosis has been proposed to use the death receptor signaling pathway by a not fully understood mechanism. To investigate the importance of functional death receptor signaling in interferon induced apoptosis, we tested whether Fas Ligand-resistant variants of the human monocytic cell line U937 showed resistance to interferon induced apoptosis. The U937 cell line was made resistant by growing the cells in increasing concentration of Fas stimulating antibody. From the resistant bulk culture 38 single clones were isolated, which all showed lower sensitivity to interferon induced apoptosis. This demonstrated that there exists signaling molecules shared by interferon and Fas-induced apoptosis, which are dysfunctional in the resistant cells. An interesting pattern of crosstalk between FasL, Trail, TNF-α and interferon induced apoptosis was identified among the resistant clones. Lower expression of Fas or impaired activation of caspase-8 was the two major phenotypes of FasL resistant cells. Interferon treatment did cause a small up-regulation of the Fas protein. However inhibition of the interaction between Fas-FasL or DR5-Trail did not abolish interferon induced apoptosis. In summery, this suggests that ligand induced aggregation of the death receptors is not needed for the induction of interferon induced apoptosis but that an intact Fas signaling pathway and normal levels of Fas are required.

14-16/P

TGF-BETA ENHANCES ADHESION OF HAIRY CELLS TO BONE MARROW FIBROBLASTS AND PROLONGS SURVIVAL OF THE LEUKEMIC CELLS

Medhat Shehata, Dita Demirtas, Susanne Schnabl, Martin Hilgarth, Rainer Hubmann, Ulrich Jaeger, Josef D. Schwarzmeier

Medical University of Vienna, Internal Medicine I, Hematology Department, L. Boltzmann Institute for Cytokine Research, A-1090, Vienna, Austria

Bone marrow (BM) infiltration with the malignant hairy cells (HCs) is a characteristic finding in patients with hairy cell leukemia (HCL). In the BM, the hairy cells are found in association with fibroblastic cells and surrounded with fine reticulin meshwork. Such a selective tissue localization may allow the HCs to receive signals for survival and proliferation. We investigated the role of TGF-beta1 in regulating the interaction between HCs and BM fibroblasts (BMF) *in vitro*.
Peripheral blood mononuclear cells from HCL patients or purified HCs were co-cultured with BM fibroblasts. Microscopic examination demonstrated a striking adhesive property of the HCs to BMF. Within few minutes, HCs were observed to adhere to and migrate beneath the fibroblasts. The adhesion of HCs to BM fibroblasts was associated with the formation of homotypic cell aggregations and enhanced survival of the leukemic cells. Under the same culture conditions, cells from HDs remained in suspension. Adhesion assays revealed that TGF-beta1 further increases the adhesion of HCs to BM fibroblasts while neutralizing anti-TGF-beta1 antibody significantly inhibits this process. To get further insight into the interaction between HCs and BM fibroblasts, we investigated the pattern of expression of adhesion molecules on the HCs. FACS analysis revealed a high expression of CD49d (VLA-4) and CD49e (VLA-5) on the HCs. Immunofluorescence studies in co-cultures demonstrated the co-localization of VLA-4 and VLA-5 with their counterreceptots CD106 (VCAM-1) and fibronectin respectively. Preincubation of the HCs with antibodies against the alpha-4 chain of VLA-4 integrin or preincubation of BM fibroblasts with anti-VCAM-1 (CD106) Ab inhibited the adhesion of HCs.
We conclude that the adhesion of hairy cells to bone marrow fibroblasts through binding of VLA-4 integrin to its ligand VCAM-1 may represent a key step in bone marrow infiltration with the malignant cells and that TGF-beta1 may promote this process.

14-17/P

PLATELET-ACTIVATING FACTOR-INDUCED NF-KB ACTIVATION ENHANCES VEGF EXPRESSION THROUGH A DECREASE IN P53 ACTIVITY

Ko HM[1], Kang Y[1], Kim HA[1], Park SJ[1], Kim KJ[1], Lee HK[2], Im SY[1]

[1]Department of Biological Sciences, Chonnam National University, Kwangju 500-757; [2]Department of Immunology, Chonbuk National University Medical School, Chonju 561-180, Republic of Korea.

We investigated the role of p53 in nuclear factor (NF)-kB dependent, platelet-activating factor (PAF)-induced vascular endothelial growth factor (VEGF) expression. Transfected NF-kB subunits in ECV304 cells increased the tumor necrosis factor- promoter activity, which was completely inhibited by p53. Transfected p53 increased p53RE promoter activity, which was completely inhibited by NF-kB subunits, indicating that cross-regulation occurs between NF-kB and p53. PAF-induced increase in VEGF expression was correlated with decreased p53 activity. These data suggest that NF-kB-dependency of the PAF-induced increase in VEGF expression is due to decreased p53 activity, which is reciprocally regulated by increased NF-kB activity.

14-18/O

LYMPHOID MICROENVIRONMENT SUPPORTS SURVIVAL OF B-CLL CELLS: ROLE OF TGF-BETA AND PI3-K/AKT PATHWAY

Medhat Shehata, Dita Demirtas, Susanne Schnabl, Martin Hilgarth, Rainer Hubmann, Markus Duechler, Ulrich Jaeger, Josef D. Schwarzmeier

Medical University of Vienna, Clinic of Internal Medicine I, Department of Hematology/Hemostaseology, L. Boltzmann Institute for Cytokine Res., K. Landsteiner Institute for Cytokine and Leukemia Res. A-1090, Vienna, Austria

The accumulation of the malignant B cells in chronic lymphocytic leukemia (B-CLL) appears to be due to inhibition of apoptosis and long survival of the leukemic cells. This could be due to activation of anti-apoptotic cascades through interaction with the lymphoid microenvironment. The aim of this study was to elucidate the role of tumor microenvironment in activation of the potent anti-apoptotic PI3-K/Akt signal transduction pathway and prolongation of survival of B-CLL cells. Stromal fibroblasts of bone marrow (BMFs) were used as an *in vitro* model for lymphoid microenvironment and to test their ability to inhibit spontaneous apoptosis of B-CLL. Co-cultivation of B-CLL cells with human BMFs significantly inhibited apoptosis of B-CLL cells. Trans-well culture experiments indicated that the supportive effect is provided by cell-cell contact and soluble mediators produced by viable fibroblasts. Experiments using TGF-beta1 or neutralizing anti-TGF-beta1 antibodies showed that this cytokine inhibits apoptosis of B-CLL cells in presence of BMF. To explore the involvement of PI3-K/Akt pathway in the anti-apoptotic effect of stromal fibroblasts, co-cultures were performed in presence of PI3-K inhibitors (wortmannin or LY294002) or siRNAs against PI3-K (p110 subunit) and Akt1. These inhibitors significantly reduced the supportive effect of stromal fibroblasts and induced apoptosis in B-CLL cells. Induction of apoptosis was associated with a significant decrease in the intracellular PIP3, PI3-K, PDK1 and Akt1, NF-kappa B, IKK, and de-phosphorylation/activation of tumour suppressor protein PTEN. Studies using phosphospecific anti-PTEN antibody demonstrated that PBMC of CLL patients (n = 40) highly express a phosphorylated (inactive) form of PTEN. The results demonstrate that PI3-K/Akt pathway is involved in inhibition of apoptosis of B-CLL cells and suggest that interaction of the leukemic cells with lymphoid microenvironment maintains the activation of this pathway. The data also suggest that targeting this pathway represents a new therapeutic approach in B-CLL.

14-19/P

GENERATION OF NOVEL TRAIL FUSION PROTEINS WITH SUPERIOR TUMOR SELECTIVE APOPTOSIS INDUCTION

Schneider B[1], Gerspach J[1], Münkel S[1], Wajant H[2], Scheurich P[1], Pfizenmaier K[1]

[1] Institute of cell Biology and Immunology, University of Stuttgart, Stuttgart, Germany; [2] Department of Molecular Internal Medicine, Medical Clinic and Polyclinic II, University of Wuerzburg, Wuerzburg, Germany

In order to improve the tumor selectivity of proapoptotic TNF ligands such as TRAIL, generation of fusion proteins comprising a recombinant antibody fragment for tumor targeting is a currently exploited strategy. These TNF family ligand fusion proteins are homotrimers, sometimes even larger assemblies, which can be prone to aggregation due to the multivalency of the single chain variable fragment (scFv) domain. For TRAIL fusion proteins this would be disadvantageous as aggregated TRAIL has been claimed to be responsible for apoptosis induction in normal tissue. We here describe a novel format of a TRAIL fusion protein, differing from the existing ones such that it is a single polypeptide in which the TRAIL module is repeated threefold to allow intramolecular folding in a way resembling a homotrimer, thus constituting a single chain (sc) TRAIL. At the N-terminus of this scTRAIL different scFv modules targeting a selective tumor or stroma marker (erbB2 and FAP) were added. This novel fusion protein is expressed as a protein of about 100 kDa molecular mass, a size compatible with prolonged half life in circulation, yet small enough to penetrate tissue. Apoptosis assays revealed TRAIL activity on TRAILR1 and TRAILR2 expressing cells. Binding of the fusion protein to tumor cells *via* scFv mediated specific recognition of the target antigen resulted in a striking increase in tumor cell sensitivity towards TRAIL induced apoptosis. Moreover, when compared to a conventional, noncovalently assembled homotrimeric TRAIL fusion protein targeting the same erbB2 epitope, the scTRAIL fusion protein showed about 10 fold higher apoptotic activity. This difference in apoptotic activity of the scTRAIL *versus* the homotrimeric TRAIL fusion protein might be explained by lack of triggering the targeted receptor tyrosine kinase by the monovalent scTRAIL fusion protein, whereas a concomitant activation of erbB2 was observed by the conventional, multivalent TRAIL fusion protein.

14-20/O

INTERFERON-α PRODUCING PLASMACYTOID DENDRITIC CELLS REGULATE T CELL FUNCTIONS IN THE ATHEROSCLEROTIC PLAQUE

Niessner A[1], Sato K[1], Chaikof E[2], Goronzy JJ[1], Weyand CM[1]

[1]Department of Medicine, Kathleen B. and Mason I. Lowance Center for Human Immunology; [2]Department of Surgery, Division of Vascular Surgery, Emory University School of Medicine, Atlanta, Georgia, USA

Plasmacytoid dendritic cells (pDC) are the main source of interferon (IFN)-α and are crucially involved in protective immune responses. We have explored the hypothesis that pDC are present in the atherosclerotic plaque providing immunoregulatory function related to plaque vulnerability. $CD123^{high}$ pDC were found in 53 % of carotid atherosclerotic plaques by immunohistochemistry (n = 30). Two-color imaging confirmed that IFN-α is primarily produced by $CD123^{high}$ pDC. Quantitative RT-PCR showed enhanced IFN-α transcripts in plaques compared to normal carotid arteries ($P < 0.00001$). IFN-α mRNA was particularly abundant in unstable plaques (defined by the presence of thrombi, lipid content, and inflammatory infiltrates) compared to stable plaques ($P = 0.00003$). pDC in plaque tissue were responsive to Toll-like receptor 9 (TLR9) stimulation. Triggering of TLR9 with oligodeoxynucleotides containing particular CpG motifs resulted in a more than twofold increase of IFN-α mRNA in plaque tissue ($P = 0.03$) and IFN-α in supernatant ($P = 0.007$). IFN-α transcripts closely correlated with TRAIL, a TNF-like ligand implicated in vascular smooth muscle cell (VSMC) apoptosis ($r = 0.67$, $P = 0.00003$). IFN-α functioned as a strong inducer of TRAIL mRNA (15.9-fold increase, $P = 0.009$) and TRAIL cell surface expression (n = 31, 10-fold increase, $P < 0.00001$) in peripheral CD4 T cells. Plaque-derived CD4 cells showed an even higher upregulation of TRAIL after stimulation with IFN-α. IFN-α-stimulated CD4 cells more efficiently induced apoptosis of VSMC than did unstimulated CD4 cells (n = 10, 43 % *versus* 32 %, $P = 0.0003$). Apoptosis could be inhibited by blocking TRAIL with specific antibodies (26 % *versus* 44 %, $P = 0.0002$). In conclusion, pDC are present in inflamed atherosclerotic plaque. In the tissue, they respond to bacteria-derived macromolecules and secrete IFN-α. Our data suggest a model of plaque injury in which TLR9 ligands destabilize the plaque by triggering pDC to release IFN-α, resulting in the induction of TRAIL on T cells and T cell-mediated VSMC apoptosis.