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[16.00-16.30]
Pathway of antibody SAM-6 induced lipo-apoptosis
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Lipids are essential for normal and malignant cell during growth and differentiation. The turnover is strictly regulated, because an uncontrolled uptake and accumulation is cytotoxic and can lead to lipo-apoptosis, lipoptosis. The human monoclonal antibody SAM-6 binds to a cell surface receptor on malignant cells. Additionally SAM-6 binds to apo-B100 on the lipoproteins vLDL and LDL, the responsible ligand for the receptor-mediated cellular uptake and catabolism of LDL. Co-incubation of malignant cells with SAM-6 and lipoproteins induces an excess of intracellular lipids, depots of cholesterol and triglyceride esters, followed by lipo-apoptosis. Interestingly, the deadly effect of SAM-6 can be increased by addition of oxidized LDL (oxLDL). This lipid over-accumulation is tumor-specific, non-malignant cells do neither bind the antibody nor harvest lipids after incubation with it. Since for both forms of apoptosis, the death-domain-dependent ("extrinsic") and -independent ("intrinsic"), the activation of proteases is crucial, we investigated the SAM-6 induced apoptotic pathway in more detail. It was found that shortly after binding, the antibody-LDL-receptor complex is internalized and depots of lipids occur. This is followed by a cytochrom c release from mitochondria and an activation of initiator-caspases 8 and 9 as well as effector-caspases 3 and 6. The mechanism of mitochondrial trigger, e.g. by free fatty acids (FFA), is under investigation. However, the present data indicate that the SAM-6 antibody induces an "intrinsic"-like form of apoptosis, by overfeeding malignant cells with lipoproteins.

[16.30-17.00]
N-glycosylation in the immunoglobulin variable region is a potential survival strategy for human follicular lymphoma cells
Kathy Potter
University of Southampton, UK

Abstract not received.

[17.00-17.20]
Characteristics of tumor gangliosides revealed by B cells infiltrating human breast carcinomas
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Introduction and background: Our project was aimed to explore the use of tumor-infiltrating B (TIL-B) cells as a source of recombinant antibodies for key tumor associated target antigens. We could generate single chain Fv immunoglobulin fragments (scFv) with unique ganglioside GD3 specificity on invasive ductal and medullary breast carcinomas by our novel strategy (1,2). Infiltrating T (TIL-T) cells (3) are well documented in solid tumors in comparison to B cells (4). EBV transformation and antibody engineering technology made it possible to clone and characterize antibodies and their fragments (5, 6). The presence of immunocompetent B cells in solid tumors may reflect ongoing immune responses against transformed cells (7). In high grade medullary breast carcinoma (MBC) the accumulation of B lymphocytes and

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plasmocytes appears to be correlated with reduction of tumor size and a relatively better prognosis (8).

The target antigens revealed by TIL-B cells turned out to be special (GD3) gangliosides, that are overexpressed on the cancerous tissue. Gangliosides are glycosphingolipids containing sialic acid residues, are mainly localized in the outer leaflet of the plasma membrane. Gangliosides have key role for important physiological and pathological cellular events, such as growth, differentiation, neoplastic transformation, adhesion, migration and invasion of tumor cells (9, 10). While gangliosides of different types are strongly expressed on tumors: (e.g.: GD2 in neuroblastomas, GD3 in melanomas, GM3 and GD3 on breast carcinomas), normal cells show negative or faint reaction with relevant anti ganglioside antibodies. Gangliosides may serve as useful target antigen for antibody mediated immunotherapy (11, 12). As it is diffi-cult to get human anti-GD3 antibodies because of their little immunogenicity, our novel way to develop antibody fragments with this specificity on breast carcinomas seems to be of great interest.

Methods. Tissue and cell samples: Tissues from patients with different types of breast carcinomas were processed, and immunohistological characteristics were defined. Tumor cell binding capacity of our TIL-B soluble scFv fragment and other relevant antibodies were tested by enzyme labelled immunoassay (ELISA), immunofluorescence FACS analysis and chamber slide culture / confocal laser microscopy. Tumor cell lines of different origin (MDA-MB 231, MCF7, SKBR3, SK-Mel 28, PANC1, LS174T, LANOAN, OVARIAN) were tested and compared to control cells (COS7).

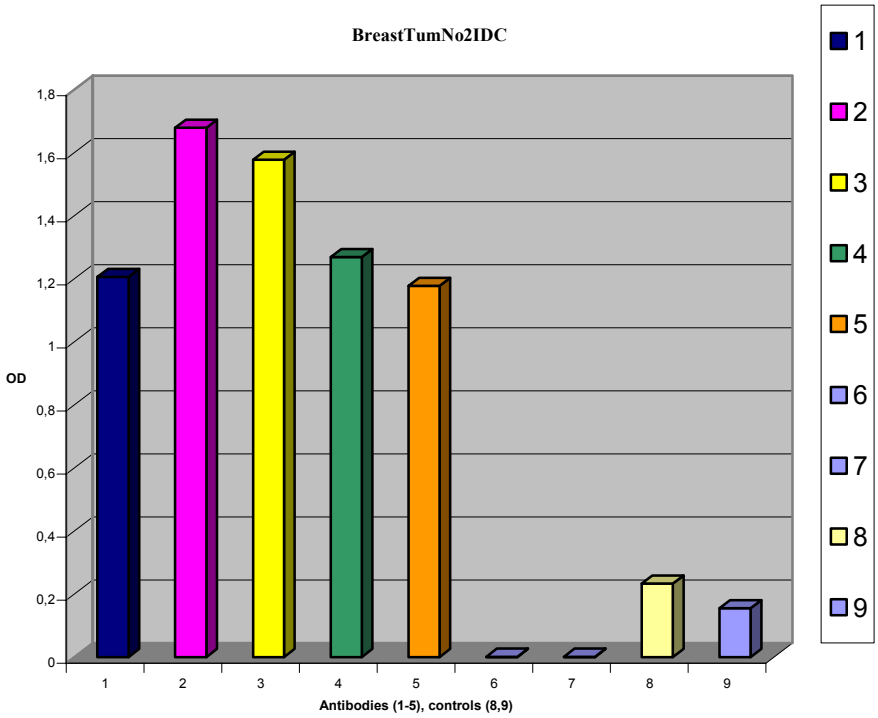

Fig 1. ELISA with soluble scFv and control antibodies: Invasive ductal breast carcinoma. cells (Tu No2) were tested. Optical density of one out of three parallel experiments is shown. 1/ MDApan sol scFvK library, 2/ G2 sol ScFvK, 3/ B2 sol scFvK, 4/ anti muc scFv, 5/ HCBC3 sup, 8/ medium control, 9/ test background.

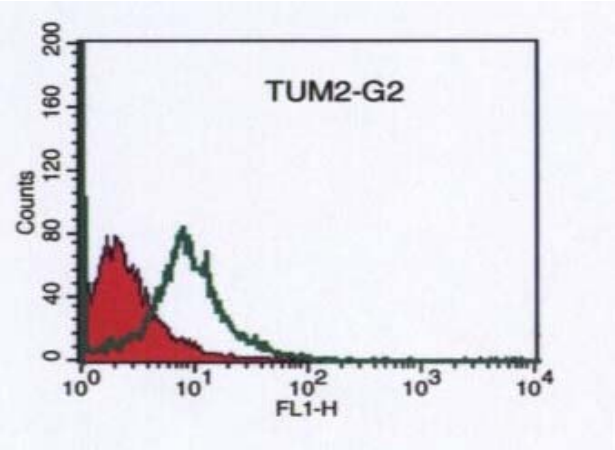

Fig. 2. ELISA with soluble scFv and control antibodies: MDA MB 231 breast cancer cell lines were tested. Optical density of one out of three parallel experiments is shown. 2/ G2 sol ScFvK, 3/ B2 sol scFvK, 4/ anti muc scFv, 5/ HCBC3 sup, 8/ medium control, 9/ test background.

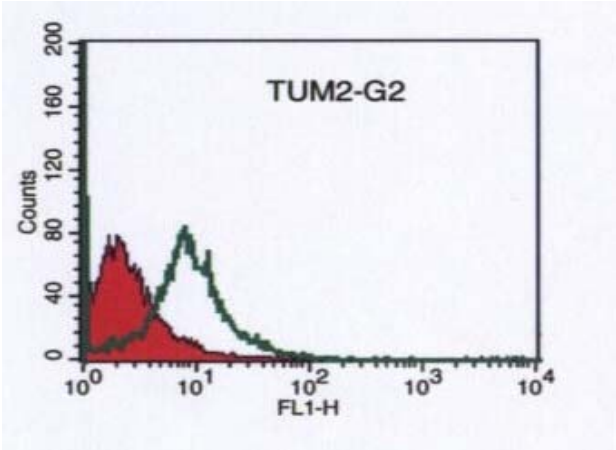

Fig. 3. Immunofluorescence FACS analysis: Specific binding of G2 soluble scFv immunoglobulin fragment was shown against cultivated invasive ductal breast carcinoma cells (Tu No2). Fluorescence intensity histogram (FL1 fluorescence intensity / cell number) is shown.

- -*Soluble scFv ELISA* was used to detect the binding capacity to different breast tumor cells: Maxisorp microtiter plates, coated with 1-10 □g of membrane preparations of tumor and control cells were blocked with bovine serum albumin (BSA). Soluble ScFv was produced by IPTG induction of bacterial clones and reacted with the coated plates. Alkaline phosphatase (AP) conjugated anti-myc antibody (Sigma) and p-Nitrophenyl phosphate (p-NPP, Sigma) subtrate system were used for detection, and the reaction was evaluated at 405 nm.
- *Immunofluorescence FACS analysis* with soluble scFv fractions: Tumor cells were incubated with soluble scFv Ig fraction and anti-c-myc 9E10 monoclonal antibody before reaction with fluorescein isothiocianate (FITC) labelled anti-mouse IgG (Fab')2. Formalin fixed cells (10000) were counted in a FACS Calibur (Becton Dickinson) and analysed by CellQuest. Confocal laser microscopy was used parallely on chamber slides.

Dot-blot analysis of gangliosides: Different gangliosides (GD1a, GD1b, GD2, GD3, GM1, GM2, GM3) (Calbiochem) and membrane preparations of breast tumor cells were dried on silica plates. Test antibodies were reacted and AP conjugated anti-myc antibody in PBS-BSA (1%) and BCIP/NBT substrate (Sigma) were used for analyzing the binding capacity. Ganglioside patterns of different ganglioside specific antibodies were compared in dot blot technique and thin layer chromatography (TLC).

Results: Our soluble scFv immunglobulin fragments revealed unique weak, less antigenic GD3 ganglioside structures on invasive ductal breast carcinomas. The highest GD3 ganglioside positivity was found with fresh cultivated cells (Tum No2) (Fig 1) of invasive ductal breast carcinoma and MDA-MB231 cell lines (Fig. 2). Our anti GD3 antibodies showed less ganglioside expression on MCF7 cells and a hardly detectable reaction in the case of SKBR3 cell line. Anti muc scFv and anti ganglioside hybridoma supernatants (HCBC3) were used as positive controls. Low levels of medium and test control sample ODs proved an effective binding capacity. The TIL-B immunoglobulin fragment showed positiv binding to breast tumor cell suppensions in indirect immunofluorescence assay (FACS analysis) and chamber slide technique (Confocal laser microscopy) (Fig.3). The reactivity pattern of the specific immunoglobulin fragments against cancerous and control tissues represented selectivity. Expressed immunoglobulin variable gene regions with different ganglioside capacities were sequenced and compared. Comparative DNA sequence analysis of different GD3 ganglioside specific antibody variable regions reflected the similarities and discrepancies of the binding capacities.

Conclusion: The impact of our novel technology is to reveal and characterize novel tumor associated GD3 gangliosides on breast cancers. Our novel approach provided a useful source to produce antibody fragments with improved properties for potential tumor targeting *in vitro* and *in vivo*. Our GD3 ganglioside

specific antibody fragment labels the more aggressive MDA-MB 231 cells, that lack the estrogen receptors (ER) and are not responsive to estrogen and anti-estrogens (tamoxifen and benzothiophene). Gangliosides are overexpressed cancer restricted molecules, and both GM3 and GD3 are suggested to be involved in the regulation of growth factor functions and tumor cell proliferation. So they are promising targets of our specific antibodies in the field of breast cancer research. The fact, that in malignant lesions an abnormal distribution pattern of O-acetylated disialogangliosides (GD3) might be defined in comparison to a benign proliferation, renders the ganglioside a putative prognostic and diagnostic marker. The identified tumor associated antigens might be used in the development of new tumor diagnostic and immunotherapeutic drug discovery programs.

Acknowledgements: The work was supported by grants to Dr B. Kotlan (OTKA T048933, OTKA T030380, NATO CLG 978639) and by the Rajko Medenica Research Foundation.

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[17.20-17.40]

Generation and application of a panel of phospho-specific antibodies against 13 *in vivo* phosphorylation sites of the tumor suppressor RB protein

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The tumor suppressor RB protein (pRB) plays a critical role in the regulation of cellular proliferation and differentiation, and it is frequently inactivated by mutation in a variety of human cancers. It is phosphorylated cell cycle-dependently and phosphorylation starts at G1/S boarder. It has been shown that its phosphorylation at G1/S boarder is first performed by Cdk4-Cyclin D and then by Cdk2-Cyclin E. The physiological meaning of this two step phosphorylation was, however, unknown. We have previously shown that Cdk4-Cyclin D and Cdk2-cyclin E have different substrate specificities using phospho-specific andtibodies. Subsequently, we have generated almost all antibodies to recognize 13 *in vivo* phosphorylation sites of pRB. Among those, 3 are monoclonal antibodies. Using these phospho-specific antibodies, we asked the physiological meaning of the two step phosphorylation of pRB at G1/S boarder.

It was found that 3 sites including Ser780 are preferred sites by Cdk4-Cyclin D1. In contrast, 3 different sites are preferred by Cdk2-Cyclin E and others are phosphorylated by both Cdk4-Cyclin D1 and Cdk2-Cylin E. We hypothesized that Cdk4-specific sites are used for release of E2F and that Cdk2-specific sites are used for release of RB-biding proteins which have LXCXE motif. This hypothesis was tested by immunoprecipitation by antibodies against E2F1 and LXCXE proteins including Brg1 and RBP1. Brg1 is a chromatin remodeling protein and RBP1 recruits histone deacetylase. Immunoprecipitates were subjected to Western blotting by phospho-

specific antibodies of pRB. Reverse experiments were also carried out. The results obtained were consistent with our hypothesis.

We further studied the phosphorylation of pRB after DNA damage. It was found, unexpectedly, that phosphorylation of only Ser612 is enhanced while all other sites are dephosphorylated after DNA damage. Moreover, this phosphorylation stimulated the complex formation between pRB and E2F-1. We have also identified the kinase to phosphorylate Ser612 as Chk1 and 2.

[17.40-18.00]
Targeting axon guidance molecules in tumor biology
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Neuropilins are both receptors for semaphorines, which are mediates of neuronal guidance, and for VEGF, an angiogenesis factor. NRP1 KO shows sever vascular defect, NRP2 KO shows lymphatic developmental defects. However the role of NRP in numerous VEGFA activities remains unclear. We have generated a panel of functional blocking antibodies that bind to both human and murine NRP1 and NRP2 from Genentech's synthetic phage library. Anti-NRP1^{sema} ~~AND~~ anti-NRP2^{sema} completely block class III semaphorine induced neuron repulsion. Anti-NRP1^{VEGF} and anti-NRP2^{VEGF} completely block the binding of VEGFA to NRP1 and NRP2 and VEGF induced endothelial cell migration, surprising so does Anti-NRP1^{sema}. In xenograft studies, it has shown that our phage antibodies slow MMTV Her2 Founder 5 tumor growth. Anti-NRP1^{sema} and anti-VEGF suggest an additive effect. Our results suggest that out anti-NPR antibodies could potentially enhance Avastin^{TM} function.

[18.00-18.30]
Cancer immunotherapy'
Zdenka L. Jonak
GlaxoSmithKline, King-of-Prussia, PA, USA

Abstract not received.

[[18.30-19.00]
[Keynote Lecture]
Antibody response in ageing humans
Donald Capra
OMRF, OH, USA

Abstract not received.