Thesis for doctoral degree (Ph.D.)

In vitro drug sensitivity test in the individualized anti-cancer chemotherapy

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Introduction

Despite the application of aggressive combined chemotherapy, irradiation, bone marrow transplantation and supportive treatment, many patients die because of the therapy resistant disease or the chemotherapy-related bone marrow depression and cytopenia resulting in severe infections and bleeding . The aim of the modern anti-cancer therapy is to increase the number of survivors, and to provide a better quality of life for the survivors using a more effective but less toxic treatment. The effectiveness of the chemotherapy does not seem to be enhanced further by increasing the aggressivity of the chemotherapy. The proper solution may be the development of a risk adopted differentiated and individualized treatment. There are two different possible ways available to develop new treatment strategies:

1. Determining the drug sensitivity of tumor cells, based on the results selecting the possibly most effective drugs and/or drug combinations for the therapy. The result of the in vitro drug sensitivity of cancer cells can be used as a prognostic factor.
2. To enhance bone marrow regeneration in order to avoid cytopenia and shorten the cytopenic period. There are favourable experiences known with the use of growth factors in clinical practice (G-CSF, granulocyte-colony stimulating factor; GM-CSF granulocyte-monocyte-colony stimulating factor), but the optimal time and dose of their application and the patient groups treatable in this way still need to be determined.

The biological properties, genetic abnormalities of tumor cells influence on their sensitivity to cytostatic drugs. It is widely known that there can be significant differences both in genetic features as well as in drug resistance profile of individual tumors with the same phenotype. This observation underlines the importance of the differentiated therapy. The final aim can be in the future to set individually determined treatment for each patient with cancer based on the individual properties of tumor cells.

In the present study we intended to develop a new drug sensitivity test which makes possible to assay numerous drugs parallel using low number of cells at the same time. We worked out three different in vitro models to study how the results of in vitro drug sensitivity could be applied in the development of clinical protocols.

1. Screening of lymphoblastoid cell lines (LCLs) seemed to be a good in vitro model in order to investigate the drug sensitivity of EBV associated lymphoproliferations.

2. In vitro testing of NK (natural killer) cell mediated killing - using isolated human NK cell - was probed to predict the effect of NK cell based immunotherapy when it was combined with chemotherapy.
3. As a third model a myeloid leukaemic cell line was chosen to study the effect of granulocyte colony stimulating factor (G-CSF) on the drug sensitivity of leukaemic cells.

Properties and treatment of the posttransplant lymphoproliferative disorder

Malignant B-cell lymphomas developing after organ transplantation can be considered as a significant side effect of the immunosuppressive treatment required for the successful graft survival. The oncogenic Epstein-Barr virus (EBV) is the etiologic agent in over 90% of cases of posttransplant lymphoproliferative disorders (PTLD) and AIDS related immunoblastic lymphomas (ARL). The overall mortality for PTLD often exceeds 50%. EBV is a ubiquitous human herpesvirus that persists for life. EBV-encoded transformation-associated proteins drive the proliferation of B lymphoblasts in PTLDs and in immunodeficiency syndrome-associated immunoblastic lymphomas. The EBV-transformed cells express nine latency-associated viral proteins: EBNA1-6, LMP-1, -2A and -2B. This latency program referred as the type III latency can be found in the *in vitro* proliferating lymphoblastoid cell lines (LCLs) that can be generated by the infection of normal human B cells with EBV. EBV drives the proliferation of human B cells *in vitro* and during primary infection *in vivo.* Strong T cell-mediated immune response has been documented *in vitro* against EBV encoded latent proteins along of a wide range of HLA class I molecules and several peptide epitopes in these viral proteins have been identified. EBV associated lymphoproliferative disease may develop only in the absence of competent immune surveillance provided by cytotoxic T cells. In SCID mice inoculated intraperitoneally with peripherial blood lymphocytes (PBL) derived from EBV-seropositive donors or with human LCLs, EBV-induced human lymphoproliferations develop within a few weeks. These lymphomas are classified as immunoblastic lymphomas, often with plasmacytoid features. A very similar phenotype can be observed in tumors of immunocompromised patients or experimental tumors growing in immunodefective mice as well as the *in vitro* growing LCLs. All the three tumor types above express the same spectrum of cell surface markers, B cell activation antigens and adhesion molecules. All of them have a normal karyotype and show identical viral gene expression patterns. The risk of development of PTLD has been found to depend upon the type of the transplanted organ, the

immunosuppressive regimen, the age, the underlying illness and the EBV status of the recipient at the time of transplantation. No controlled studies have been taken in the management of PTLD and most of the recommendations for its therapy come from small cohorts at single institutions. The relative importance of T cell impairment, EBV and clonal proliferation has led to the following strategies:(i)reduction of immunsuppression or prophylactic restoration of T-cell immunity, (ii) introduction of antiviral therapy and chemotherapy. Anti-B-cell monoclonal antibodies proved to be an effective therapy for PTLD. The response rate of rituximab (humanized monoclonal antibody against the pan-B cell marker CD20) treatment is 65% with a relapse rate of 18%. Patients who do not respond to the therapy mentioned above, are treated with chemotherapy as a second or third line of option. The most frequently used chemotherapy regimes are the following:: (i) the anthracycline based CHOP (cyclophosphamide, doxorubicin, vincristine and prednisone),(ii) VAPEC-B (doxorubicin, etoposide, cylophosphamide, methotrexate, bleomycin, and vincristine); (iii) VACOP-B (cyclophosphamide, vincristine, bleomycin, prednisone, with increasing doses of doxorubicin and etoposide) or (iv) DHAP (cisplatinum, cytosine-arabinoside, dexamethasone).

Remission rate for anthracycline-based combination therapy is 69%. Chemotherapy of PTLD is associated with a rather high mortality rate due to the treatment-related toxicity. Chemotherapeutic agents can also compromise the survival of the graft. Drugs that are effective even at a low dose, also providing a better graft survival with less side effects would be preferred.

The combination of natural killer cell based immunotherapy with chemotherapy

The goal of immune-based adjuvant therapeutic strategies is to stimulate the immune effector cells to kill malignant cells resulting in a better clinical outcome. Infiltration of solid tumors with inflammatory cells is a well known phenomenon. Several studies showed that the infiltration of malignant tumors with natural killer (NK) cells was associated with a better clinical outcome. NK cells are large granular lymphocytes that are part of the innate immune system. These cells have the ability to directly kill target cells and produce immunoregulatory cytokines in transfused cancer patients. The outcome of NK cell-target cell interactions is regulated by a fine integrative balance between the inhibitory and activating receptors. Since NK cells are able to spontaneously lyse tumor cells considerable effort has been focused on investigating the possible use of NK cells in the treatment of human cancer. Recent

understanding of NK cell development and function has permitted the investigation of novel NK cell–based immunotherapeutic modalities. A promising approach is to expand allogeneic NK cell clones *in vitro* and to transfuse these cells into leukemic patients. NK cells have also been used in human therapeutic regimes including the administration of high or low doses of IL-2 alone or in combination with IL-2–activated blood lymphocytes or purified populations of NK cells for primary lung and renal cell carcinomas. In other studies an extended low-dose IL-2 was used to activate NK cells in patients with solid tumors who received intensive chemotherapy or in those with acute myeloid leukemia. The chemotherapeutic regimes for malignancies restrict the effectivity of the immun system and significantly decrease the level of NK cells. The activity of NK cells is also affected by some cytostatic drugs. An enhanced NK cell-mediated cytotoxocity was observed after treatment of target cells with topoisomerase inhibitors. Killing by NK cells requires microtubule integrity which may be influenced by anti-microtubule agents. It is important to consider the drug induced effects when chemotherapy and NK cell mediated immunotherapy is planned to be used in parallel.

G-CSF in the adjuvant treatment of acute leukaemia

Leukemia is the most common cancer in childhood, accounting for about 30% of all childhood malignancies. The major morphological subtypes of leukemia in childhood is acute lymphoblastic leukemia (ALL), and acute myeloblastic leukemia (AML). About 80% of ALL patients in childhood can be cured due to agressive chemotherapeutic protocols, improved supportive treatment, application of various biological modulators and bone marrow transplantation. However, 20% of ALL and about 50% of AML patients still die because of resistance to therapy and/or side effects of agressive chemotherapy. The most common cause of death is represented by cytopenia-related complications such as bleeding and infections. Febrile neutropenia is a potentially life threatening side effect of the current chemotherapy. Several clinical trials have attempted to evaluate the effect of colony-stimulating factors (CSF) in shortening the period of neutropenia and preventing febrile period in children with solid tumors, ALL and with some types of AML, but in acute leukaemia the benefit of growth factors (GFs) for the patients' overall survival has not been proved. Haemopoietic GFs such as granulocyte-macrophage colony-stimulating factor (GM-CSF), erythropoetin (Epo) and granulocyte colony-stimulating factor (G-CSF) are widely used in clinical practice. G-CSF is known to induce proliferation and differentiation of normal myeloid precursors. Binding of G-CSF to its receptor leads to receptor activation, signalling through JAK 1 and 2, and

internalization of the receptor-cytokine complex. G-CSF receptor has been shown to be expressed on normal myeloid lineages. In vitro G-CSF stimulates the formation of granulocyte colonies, induces proliferation, inhibits apoptosis and commits these cells to differentiation into the neutrophil polymorph lineage. The in vitro responsiveness of AML cells to G-CSF has been extensively studied. According to these data, in the early period of application of G-CSF in pediatric oncology, its use in myeloid malignancies was contraindicated. However, several double-blind clinical trials demonstrated that G-CSF administration did not induce blast proliferation in AML patients. Based on these findings G-CSF was used in clinical trials to prevent neutropenia in AML and for priming (driving AML cells into cell cycle and to increase the proportion of leukemic cells in S-phase) in order to increase the blasts' response to cytarabine or fludarabine, drugs predominantly affecting cells in S-phase of the cell cycle. At the same time stimulatory effects of G-CSF on leukaemic lymphoblasts are unexpected, since according to our current knowledge it does not have any influence on pathways of proliferation and differentiation of cells in the lymphoid lineage. Although, the effects of these GFs at a cellular level are not well understood, the clinical use of G-CSF to minimize chemotherapy-induced myelosuppression in childhood ALL has become more widespread.

There are several reports suggesting that some ALL cells with B- or T-cell surface antigens, or biphenotypic leukemia cells express G-CSF receptors and they are able to respond to exogenously added G-CSF. Based on these studies the question arises whether G-CSF used as a component of the supportive treatment of childhood ALL with febrile neutropenia can lead to proliferation of not only the normal myeloid cells but of lymphoid leukaemic blasts coexpressing myeloid markers as well. In addition, whether or not G-CSF influences drug sensitivity of blast cells remains to be answered, too.

Aims

The goals of our study were as follows.

1. We planned to develop a novel and higly sensitive drug sensitivity test which can be used for investigating numerous cytostatic drugs with the application of low number of tumor cells.
2. We intended to investigate the *in vitro* drug sensitivity of Epstein-Barr virus (EBV) transformed lymphoblastoid cells, and to select the in vitro most effective cytostatic drugs that have not been used till now for treatment of EBV induced lymphoproliferations. We planned to carry out over 6500 cell survival and

proliferation assays on single cell levels in order to establish the pattern of *in vitro* efficacy of the most commonly used cytotoxic drugs against EBV-transformed lymphoblastoid cells.

3. Our further aim was to investigate systematically the effect of the most frequently used cytostatic drugs on NK cell-mediated cytotoxicity. We intended to carry out over 4000 cell survival assays on single cell levels using automated laser confocal microscopy in order to establish the pattern of *in vitro* effect of 29 different cytotoxic drugs on the efficiency of NK mediated killing. We looked for drugs that had less influence on the NK mediated killing and could be used together with the NK cell applied immunotherapy.
4. We wanted to investigate the chemosensitivity of leukaemic blasts to daunorubicin after G-CSF pretreatment. Daunorubicin was chosen for our studies because this drug is widely used in ALL and AML protocols due to its marked apoptotic effect.

Materials and methods

Drug sensitivity of lymphoblastoid cell lines

Cell lines and culture conditions

Seventeen lymphoproliferative cell lines (LCL) were used in the present study. 980215, 031016, 040113, 051018, JAK, LP, LSZ, MIN, AF, GK, FUR, HA, VMB were established by *in vitro* infection of normal B lymphocytes obtained from different healthy donors with the B95.8 strain of EBV and expanded in IMDM medium supplemented with 10% fetal calf serum (FCS). LS was a spontaneus LCL established from a healthy donor. IARC171 was established using B95.8 virus infection of normal lymphocytes of a patient with Burkitt lymphoma. The LCL, TR were established from B cells of an XLP (X linked lymphoproliferative disorder) patient who had a deletion in SAP gene. The LCL, IB4 contains an integrated EBV genome.

During the time of the tests the LCLs were cultured in IMDM supplemented with 20% FCS.

Drugs

For the *in vitro* drug sensitivity test 29 drugs were used. All the drugs were dissolved in 50% dimethyl sulfoxide (DMSO) and printed on 384 well plates using high density array replicator

metall pins with 50 nL replica volumes in Biomek 2000 fluid dispenser robot (Beckman). The same robot was used to generate the drug masterplates containing the triplicates of four different drug dilutions using a single tip automatic pipettor dispenser head. The highest drug concentration was selected as the physicochemically maximally achievable drug concentration at 600 times dilution (50 nL drug in 30 µL assay volume). The Ratio of Maximum Achieved Plasma Concentration (RMAPC) was determined for each drug concentration in order to compare the *in vitro* tested concentrations to *in vivo* maximally achieved plasma concentration (C_{max}) levels. RMAPC is calculated according to the following equation:

RMAPC= *in vitro* tested concentration / C_{max}

Fluorescencent in vitro drug sensitivity assay

In vitro drug resistance of LCLs was assessed using a three-day cell culture on microtiter plates. Twenty eight drugs were tested, each at four different concentrations in triplicates on 384-well plates. Each well was loaded with 30 µL cell suspension containing 3000 cells. After three days of incubation the live and dead cells were differentially stained using fluorescent VitalDye. The precise number of living and dead cells was determined using a custom made automated laser confocal fluorescent microscope at the Karolinska Institute visualization core facility (KIVIF). The images were captured with using the computer program QuantCapture 4.0 whereas the living and dead cells were identified and individually counted using the program QuantCount 3.0. Fifteen control wells, that were used to determine the control cell survival (CCS), contained cells with only culture medium and 50 nL DMSO without drugs, five wells contained cells with culture medium alone. Comparing the two types of control wells no toxic effect of DMSO could be seen. Mean cell survival (MCS) was determined from the average of survival of all 17 LCLs.

Cytotoxicity of NK cell cultures in the presence of cytostatic drugs

Isolation and culturing of human polyclonal peripheral blood NK cell cultures.

Polyclonal NK cells were acquired from lymphocyte-enriched buffy coat derived from healthy donor blood. Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation on Lymphoprep™. NK cells were isolated from PBMCs by negative magnetic bead selection according to the manufacturer's instructions. Purified NK cells were >95% $CD3^-CD56^+$. NK cells were cultured either overnight or for several weeks in IMDM

supplemented with 10% human serum, 2 mM L-glutamine, 1× non-essential amino acids, 1mM sodium pyruvate, 50 U/mL penicillin-streptomycin, 50 μM 2-mercaptoethanol and 100 U/mL IL-2. Eight primary NK cell isolates were tested in the present study. Five NK isolates were re-stimulated every 3-4 days with fresh IL-2 containing media for one week and were periodically monitored for the $CD3^{-}CD56^{+}$ phenotype. Three NK isolates were stimulated with IL-2 only for 24h.

Target cells and culture conditions

K562 cell line was used as target in the *in vitro* killing assay. These cells were cultured in IMDM supplemented with 10% FCS.

In vitro killing assay

The effect of drugs on *in vitro* killing efficacy of different NK lines was assessed using microtiter plates. Twenty nine drugs were tested, each at four different concentrations in triplicates on flat bottom 384-well plates. Each well was loaded with 20μL cell suspension of NK cells containing 15000 cells and was incubated for 20 h. Target (K562) cells were stained with CellTracker green. As a next step 20μL of target cells (K562) containing 3000 cells were seeded on the same plate and were incubated with the NK cells for 5 hrs (effector: target, E:T ratio 5:1). Dead cells were differentially stained red using ethidium-bromide. The precise number of green target cells and red dead cells was determined by using the same custom designed automated laser confocal fluorescent microscope. The images were captured using the computer program QuantCapture 4.0 and the living and dead target cells were identified and individually counted using the computer program CytotoxCount 3.0. The target cells were identified as dead if they were stained red and green at the same time. On each plate eight wells were set up as controls to determine the baseline killing activity. These wells contained NK and K562 cells in culture medium only, without any drugs. As additional control only effector NK cells were incubated alone under the same conditions. To monitor the possible short term effect on the target cells, K562 cells were incubated alone and with all the individual drugs for five hours. Killing effectiveness (KE) of NK cells was calculated for every well using the following formula: **[number of dead target cells (both red and green signal in the same cell) / number of all (green) target cells] x 100%**.

To normalize the killing effect values of different NK populations the following formula was used:

KE in the wells with drugs / mean KE values of the controll wells x 100%.

Mean killing efficacy (MKE) was determined from the average of killing of all NK populations. The red stained cells which were not green at the same time were considered as dead NK cells. The cytotoxic effect (CE) of the drugs on NK cells was calculated with the following formula:

number of dead NK cells in the wells with drugs / number of dead NK cells in the control wells x 100%.

Drugs

For the *in vitro* killing test twenty nine drugs were used. Twenty eight of them are regularly used in the treatment of human patients. The drug plates were prepeared as it was described above. The highest drug concentration was selected as the maximum drug concentration physicochemically achievable at a 600 times dilution (50 nL drug in 30 µL assay volume). These concentrations were in a comparable range with the *in vivo* plasma concentrations of patients. The starting concentrations of the dilution series (1x, 5x, 25x, 125x) for the individual drugs were initially determined based on the solubility of the different agents.

Testing the drug plates on a large number of *in vitro* tumor cell lines and primary tumors we could show that it was possible to find sensitive cell lines for each individual drugs, demonstrating that all the drugs on the plate were active. A way to calculate a relationship between the *in vitro* drug concentrations and the *in vivo* ones is to use the area under curve (AUC) values of the individual drugs. For this comparison Ratio of Area Under Curve values (RAUC) were determined by the following formula:

***in vitro* used concentration (µg/mL) x 20 hours/ AUC (µgxh/mL) *in vivo*.**

A RAUC value higher than 1 indicates that the *in vitro* used dose is higher than the one used in clinical practice. If this value is one it means that our in vitro dose corresponds to the clinical dose.

The effect of G-CSF pretreatment on leukaemic blast cells to daunorubicin sensitivity

Cell culture

Ex vivo experiments with AML and ALL cells are hardly repeatable from the same sample. Although there are several ALL cell lines available, whether they express G-CSFR is not known, since in most of the acute lymphoblastic leukaemias myeloid markers are absent. KG-1 is a leukaemic cell line expressing myeloid markers and G-CSF receptor and thus offers a good in vitro model for testing G-CSF receptor function of both AML and G-CSFR and

myeloid markers expressing ALL. Therefore, KG-1 cell line was used to compare the chemosensitivity of cells to daunorubicin in the absence or presence of G-CSF. KG-1 cells were cultured in IMDM supplemented with 20% FCS.

Flow cytometry

KG-1 cells were cultured in AIMV serum free medium for 16 hours and used for flow cytometry measurements. KG-1 cells were stimulated with 10 ng/mL G-CSF for 48 hours alone or treated with daunorubicin (2µg/mL) for 6 hrs. The control samples were cultured in AIMV for 64 hours. Cells were harvested by pipetting, washed twice with saline and resuspended. Cells (3×10^5/mL) were labeled with fluorescent monoclonal antibody conjugates specific for: CD11a, CD11c, CD34, CD58, CD86, HLA-DR, CD11b, CD114 (G-CSF-R), MDR-1 (multidrug resistance protein-1). Control samples were labeled with isotype-matched antibodies conjugated with the same fluorochrome. Expression level of cell surface markers was measured after direct or indirect immunofluorescence labeling by flow cytometry using FACS Calibur flow cytometer.

Immunoblotting

After 16h incubation in AIMV serum free medium KG-1 cells were stimulated with 10 ng/mL G-CSF for 3, 6, 12 hours. The control samples were cultured in AIM V for 16 hours. After incubation with G-CSF, cells were washed with ice-cold PBS, scraped off and lysed in 100 µL RIPA buffer on ice and sonicated. Whole cell lysates were used for Western blot analyses. Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Samples for Western blot analysis were diluted in RIPA buffer and boiled after addition of 5x SDS sample buffer. For analysis of proteins by Western blots 70 µg protein was loaded onto the gels. The gels were blotted onto nitrocellulose membranes using a BioRad transfer unit at 100 V for 90 min with BioRad cooling units. After blocking, the membranes were washed and incubated with primary antibodies (anti- p44/p42 and anti-P-p44/42) for 90 min at room temperature or overnight at 4°C, followed by application of the HRP-conjugated secondary antibodies. Immunoreactions were detected by enhanced chemiluminescence (ECL). PBS was substituted for TBS when phosphospecific antibodies were used.

MTT assay

After 16h starving in AIMV serum free medium KG-1 cells were stimulated with 10, 100, 1000 ng/mL G-CSF for 48 hours alone or combined with daunorubicin (0,002-2 µg/mL). The control samples were cultured in AIMV for 64 hours. The use of AIMV medium instead of the commercial IMDM was necesarry for the experiments to have constant level of growth factors. Culturing KG-1 cells in AIMV for 16 hours was used to sensitize the cells to G-CSF. Cellular resistance was measured using a total cell killing methyl-thiazol tetrazolium (MTT) assay. Briefly, KG-1 cells were cultured in AIMV serum free medium. The cells were seeded to 96-well microculture plates (80 µL/well of 5 $x10^5$/mL) and after 16 hour incubation they were stimulated with 10, 100 or 1000 ng/mL G-CSF for 48 hours alone and combined with daunorubicin at 0,002-2 µg/mL concentrations. The control samples were starved in AIMV for 64 hours. Six wells contained culture medium only, and six other wells contained culture medium with cells to determine the control cell survival (CCS). MTT was added after the incubation time and it was converted by viable cells into a colored formazan product which was measured spectrophotometrically at 562 nm. The optical density (OD) was linearly related to the number of viable cells. Cytotoxicity was calculated at each drug concentration by the following equation: **(OD treated well/mean OD control wells) x 100%,** after correction for the background OD of the wells with culture medium only.

Results

Drug sensitivity of lymphoblastoid cell lines

Comparison of the drug sensitivity pattern of the different LCLs

The 17 different LCLs that were tested in the present study represent a large variety of cells with different origin and *in vitro* history. The investigation included cell lines with several years of continuous *in vitro* culturing together with freshly established transformed B-cell cultures of only 3 weeks of age. Many of the lines were transformed by the B95-8 strain of EBV but some of them were spontaneous outgrowths driven by the donors' own virus. Among the lines we had LCLs from healthy donors, from XLP patients or lines established from the normal B-cells of Burkitt lymphoma patient. Remarkably the LCLs of different origins showed a very similar sensitivity pattern against the different drugs.

Identification of highly effective and non-effective drugs

The starting concentrations of the dilution series (1x, 4x, 16x, 64x) for the individual drugs were initially determined based on the solubility of the different agents. To make the cell survival data comparable with each other first we have compiled the known Maximum Achieved Plasma Concentration data (Cmax) for all the drugs from the literature. Dividing the actual drug concentrations in the dilution series with the Cmax of the particular drug yielded the Ratio of Maximum Achieved Plasma Concentration (RMAPC). Plotting the cell survival against the RMAPC values allowed a direct comparison of the effectiveness of the different drugs. Based on their effectiveness on LCLs, we have divided the drugs into three different groups:

Group 1: The drugs were considered to be highly effective if the mean cell survival (MCS) was below 30% at RMAPC 0.3 or below it.

Group 2: The drug was partially effective if MCS was under 60% and RMAPC was between 0.3 and 1.

Group 3: The drug was ineffective, i.e.. the LCLs were resistant for the drug, if MCS was above 60% or RMAPC was above 1.

The four most effective drugs against LCLs proved to be as follows:: **vincristine, paclitaxel, methotrexate, epirubicin** (Group 1). Gemcitabin, cytosine-arabinoside, doxorubicin, fluorouracil, dactinomycin, docetaxel, daunorubicin, etoposide, vinorelbine were rated as partially effective (Group 2.) Most LCLs proved to be resistant to cyclophosphamide,

asparaginase, topotecan, oxaliplatin, bleomycin, 6-mercaptopurin, hydroxyurea, cladribine, chlorambucil, carboplatin, bortezomib, cytarabine, prednisolone and vinblastine (Group 3). Almost no drug response could be seen with oxaliplatin, bleomycin, cyclophosphamide, asparaginase, hydroxyurea and ifosphamide whereas vinblastine, chlorambucil, prednisolone and topotecan were found to be effective, but only at very high concentrations, far above the maximum achievable plasma concentrations. Although the Group 3 drugs were not effective against LCLs these drugs showed a concentration dependent growth inhibitory effect on other cell lines or primary tumors. No RMAPC could be determined for streptozocin because no pharmacokinetical trial with established Cmax plasma levels was found. RMAPC was not available for MG132 either because it is not used in clinical practice. Streptozocin had no effect on survival at the concentrations used in the study. Although at the highest concentrations the proteosome inhibitor MG132 effectively decreased the survival of cells, its clinically licensed functional homologue, bortezomib, was not effective.

Cytotoxicity of NK cell cultures in the presence of cytostatic drugs

The effect of cytostatic drugs on the killing capacity of different NK cells

The NK cells of different origins showed a very similar killing efficacy pattern in the presence of most of the drugs. Importantly, none of the drugs influenced the viability of the K562 target cells during the 5h incubation. The number of dead NK cells in the drug treated cultures that received IL-2 for only one day was higher than those in the drug treated cultures that received IL-2 for one week. None of the NK populations showed dose dependent increase in the number of dead cells. Consequently, the dose dependent decrease in the killing effect of NK cells in case of certain drugs cannot be simply explained by drug induced NK cell death.

Identification of highly effective and non-effective drugs

The most effective drugs that inhibited NK mediated killing were found to be the following: chlorambucil, MG-132, docetaxel, cladribine, paclitaxel, bortezomib, gemcitabine, vinblastine. For MG-132 no RAUC value could be defined because this drug has not been used clinically. Oxaliplatin, dactinomycin, cytarabine, daunorubicin, vincristine, topotecan were only marginally effective.

Most NK cell lines were not affected by bevacizumab, bleomycin, doxorubicin, epirubicin, vinorelbine, carboplatin, methotrexate, ifosphamide, etoposide, hydroxyurea, asparaginase, 5-fluorouracil, 6-mercaptopurine, streptozocin and cyclophosphamide.

The effect of G-CSF pretreatment of leukaemic blast cells on their daunorubicin sensitivity

Detection and activation of G-CSF-R in KG-1 cells

Expression of G-CSF-R on the surface of KG-1 cells was demonstrated by flow cytometry. It is known that G-CSF induces the activation of ERK/MAP kinase pathway, which is reflected in the phosphorylation of the ERK/MAP-kinase isoforms. To test whether G-CSF receptors of KG-1 cells are functional, G-CSF induced increase of phosphorylated ERK-1 (p44/p42 MAP kinase) was tested by Western blot analysis. The amount of total ERK-1 decreased after treatment of KG-1 cells with G-CSF. Phosphorylated ERK-1 was not detectable in the untreated sample, but after 3 hours of treatment with G-CSF phosphorylated ERK-1 (P-ERK-1) appeared. After 6 hours of G-CSF treatment the level of P-ERK-1 increased further and the extent of phosphorylation remained sustained after 12 hours.

Effect of daunorubicin and G-CSF on KG-1 cells

G-CSF added to cultured KG-1 cells at different concentrations (10, 100, 1000 ng/mL) did not change the survival of KG-1 cells. KG-1 cells proved to be sensitive to daunorubicin in the concentration range used in the in vitro experiments. Treatment of the cells with G-CSF in the presence of low concentration of daunorubicin resulted in about 20% more live cells compared to control. No difference in drug sensitivity of KG-1 cells was observed when the cells were cultured in AIMV or FCS-containing IMDM media. Treating the cells with G-CSF and daunorubicin together resulted in a dose dependent effect: after the pretreatment of KG-1 cells with G-CSF: an increase in the cell survival could be observed at the highest concentration of daunorubicin (2µg/mL). Interestingly, the increase in the survival was more apparent at the lowest concentration of G-CSF (10ng/mL).

Expression of CD markers after treatment with G-CSF and daunorubicin

To control whether the increase of daunorubicin resistance in the presence of G-CSF is the consequence of the differentiation of leukaemic cells, the phenotypic changes of G-CSF-treated cells was measured by the expression of different cell surface markers using flow cytometry: G-CSF alone (10ng/mL) did not change the expression level of any investigated markers (CD11a, CD11b, CD11c, CD34, CD58, CD86, HLA-DR). High concentration of daunorubicin (2µg/mL) alone depressed the level of expression of every investigated cell

surface marker. In the expression level of CD11a a more significant decrease was caused by daunorubicin after pretreatment with G-CSF.

Discussion

Epirubicin and paclitaxel could be effective drugs in the treatment of posttransplant lymphoproliferative disorder and in other EBV induced lymphoproliferations

The presented data suggest that many EBV transformed B-cell lines share a common cytotoxic drug sensitivity profile independent of their origin. This profile does not change even upon many years of *in vitro* culturing. EBV appears to be the necessary and sufficient etiological agent behind the malignant immunoblastic B-cell lymphoproliferations in immunosuppressed patients. In all these EBV encoded cases the, latency-associated viral proteins drive the cell proliferation without any obvious need for additional genetic changes. The phenotype of these tumors highly resembles the *in vitro* growing LCLs and the experimentally induced tumors that appeared upon intraperitoneal implantation of LCLs into SCID mice. Considering the phenotype and karyotype stability of EBV transformed B-cells, our data suggest that PTLDs, AIDS associated CNS lymphomas and XLP associated lymphoproliferations may show similar pattern of drug sensitivity to the one that we have established in the present study on a cohort of diverse LCLs. Our data show that euploid LCLs are particularly sensitive for anti-microtubule drugs and antracyclines. Although all LCLs showed a dose dependent increase of cytotoxicity when were treated with various members of these two drug families, only vincristine and paclitaxel as well as epirubicin and doxorubicin were considered to be effective when we compared the *in vitro* used drug concentrations with the maximum achievable plasma concentration levels. Alkylating agents such as cyclophosphamide and ifosphamide were not found to be active on LCLs. This might be explained by the fact that both of these compounds are prodrugs that have to be converted into active metabolites by the liver *in vivo*. Prednisolone proved to be active in very high concentrations only. Vincristine and methotrexate are included in the frequently used CHOP and VAPEC-regimes, but no data could be found for the clinical application of epirubicin and paclitaxel for the treatment of PTLD or other EBV induced lymphoproliferations. Based on our results we suggest the inclusion of epirubicin and paclitaxel into chemotherapy protocols against PTLD.

Cytostatic drugs that can be combined with NK cell-based immunotherapy

In our experimental model the killing effectiveness of NK cells was tested in the presence of different cytostatic drugs. Previous studies investigated NK-mediated cytotoxicity following treatment of target cells with certain cytotoxic drugs or treating NK cells with a few chemotherapeutic agents before performing NK killing assays. No systematic study, however, has been carried out to investigate the effect of a large number of different drugs at different concentrations. In our experimental setup the NK cells were preincubated for 20 h only with the drugs in order to test the drug effect on the NK cells. This was followed by coincubation with the target cells for 5 hours. No drug-induced cytotoxicity was detectable on target cells during this incubation, although other effects on target cells, including influence on NK killing, cannot be excluded. The presented data suggest that IL-2 activated NK cell lines share a common profile of cytotoxic drug sensitivity. This profile does not change upon different length of *in vitro* culturing with IL-2. Almost no effect could be seen in case of carboplatin, methotrexate and dactinomycin, although this might be explained by the relatively low range of RAUC levels achieved in the microcultures. Although chlorambucil efficiently inhibited the killing effect of NK cells, other alkylating agents like cyclophosphamide and ifosphamide did not affect NK killing. This might be explained by the fact that both of these latter compounds are prodrugs that have to be converted into active metabolites in vivo by the liver. The effectiveness of chlorambucil may also be explained by the high *in vitro* RAUC levels. *In vitro* doses (*in vitro* AUC levels) of the other effective drugs were in close vicinity to the clinical doses. Unlike other authors who found no inhibition of NK killing ability when only effector cells (NK) were treated with cladribine, in our experimental setup this agent was the only drug of the base analogues that significantly decreased the killing effect of IL-2 activated NK cells. Our data also showed that NK cells were particularly sensitive to anti-microtubule drugs and proteosome inhibitors. The dependence of NK cell-mediated killing on microtubule integrity is well known. Here we show that different microtubule inhibitors have a variable effect on NK mediated killing. Vinorelbine and vincristine had much less effect on NK-mediated killing than vinblastine, docetaxel or paclitaxel. Here we show that proteosome inhibitors severely compromise the killing efficiency of human NK cells even in the absence of apoptosis induction. Our results suggest that chemotherapy protocols including proteosome inhibitors or anti-microtubule drugs may interfere with NK cell based immunotherapy, if applied simultaneously. Based on the almost complete absence of inhibitory effect on NK-mediated killing even at high RAUC levels we suggest that the following drugs may be

effectively combined with adjuvant NK based immunotherapy: asparaginase, bevacizumab, bleomycin, doxorubicin, epirubicin, etoposide, 5-fluorouracil, hydroxyurea, streptozocin and 6-mercaptopurine. The data presented here, could potentially be used in the future to design new NK based adjuvant immunotherapy protocols.

G-CSF might decrease the sensitivity of leukaemic cells against daunorubicin

Colony-stimulating factors have been used to prevent febrile neutropenia and infection of patients treated with myelosuppressive drugs for cancer. It has been demonstrated in solid tumors that myeloid cytokines may be given safely to patients receiving anti-cancer chemotherapy. Due to *in vitro* data showing that G-CSF may elicit cellular proliferation of AML, clinicians have been cautious fearing acceleration of the disease process. However, double-blind clinical trials demonstrated that G-CSF administration did not induce blast proliferation in AML patients. The aims of using G-CSF in the treatment of ALL and AML are partially different. In AML G-CSFR is presented on the cell surface and it has been used for priming in order to syncronize leukaemic cells and to sensitize them against S-phase chemotherapeutic agents. G-CSF has been also given to AML patients to shorten febrile neutropenia period after induction therapy. In childhood ALL the use of G-CSF has become widespread to shorten febrile neutropenic period, since under physiological circumstances G-CSF does not have any influence on the proliferation/differentiation pathways in lymphoid lineage, therefore it is thought that G-CSF has no effect on ALL blasts.

There are several reports, however, implying that some B- or T-cell leukaemias, biphenotypic leukemia cells, or leukaemic cells with specific translocations may express G-CSF receptors as well and are able to respond to exogenous G-CSF. In the present work we studied how G-CSF in cell suspension influences the drug sensitivity of KG-1 leukaemic cells. Those acute lymphoblastoid leukaemia cells that carry G-CSFR on their surface usually co-express myeloid surface markers as well. Therefore, to investigate the effect of G-CSF the KG-1 myeloid leukaemic cell line was thought to be a good model not only for AML, but even for this special type of ALL as well. In our experimental setup pre-treatment of KG-1 cells with G-CSF followed by relatively high concentration of daunorubicin resulted in an increase of KG-1 cells survival. G-CSF alone did not change the survival of leukaemic cells, whereas in the presence of low concentration of daunorubicin the number of live cells appeared to be significantly higher compared to that of control cells, implying a moderate increase in the

viability of the cells. In our experiments, however, daunorubicin treatment led to the disappearance of multidrug resistance protein, therefore drug resistance could not be explained by the effect of this efflux pump.

Another mechanism to be considered is that binding of G-CSF to G-CSFR may induce granulocytic differentiation and leads to rapid and sustained activation of the ERK1/2 kinases. Upon G-CSF treatment of KG-1 cells we observed an increase in the level of phosphorylated ERK1/2 indicating G-CSF receptor-mediated signaling. Surface markers that could be detected on KG-1 myeloid cells have been proved to be changed during the differentiation of monocytes to macrophages. In order to follow the hypothetized cell differentation process due to G-CSF treatment the expression of several surface markers on KG-1 cells has also been studied. Daunorubicin alone depressed the level of all investigated markers. After treating the cells with G-CSF and daunorubicin together an even more prominent decrease in the expression level of CD11a was detected. The CD11a is a lymphocyte function-associated antigen-1α (LFA-1α or CD11a) and it represents the only integrin expressed on all leukocyte lineages. CD11a is known as a key adhesion receptor in immune and inflammatory processes as well as a signal-transducing molecule. LFA-1 expression and function are dependent on the state of cellular activation and differentiation. On B lymphoblastoid cells c-myc is known to cause a transcriptional and posttranscriptional down-regulation of LFA-1. Maturation of monocytes into macrophages is accompanied by characteristic changes in CD11a expression. It was also shown that G-CSF promotes cell survival mainly by suppressing apoptosis via pathways that modulate the p21ras/MAP kinase signalling pathway and by increasing the level of BCL-2 antiapoptotic protein. Our present findings show that G-CSF and daunorubicin together may act in synergy to decrease the surface expression of CD11a. Accordingly, the differentiation effect of daunorubicin coupled to the antiapoptotic signaling induced by G-CSF may combine to result in increased survival and drug resistance of the cells. In conclusion, our results point to a potential hazard of giving G-CSF to ALL patients with febrile neutropenia, especially those which coexpress myeloid markers and G-CSFR and treated with daunorubicin. Despite these findings, G-CSF could be administered individually to ALL patients with blast cells not expressing G-CSF receptors. On the other hand - despite the finding that after induction therapy the externally given G-CSF does not induce blast proliferation in AML patients - adjuvant treatment of AML patients with G-CSF in order to prevent neutropenia, or its use in priming regimens might result resistance to daunorubicin.

Most important results and conclusions:

1. New in vitro drug sensitivity method has been developed that gives possibility to investigate numerous cytostatic drugs with the application of low number of tumor cells.
2. Based on our results we suggest the inclusion of epirubicin and paclitaxel into chemotherapy protocols against PTLD.
3. Asparaginase, bevacizumab, bleomycin, doxorubicin, epirubicin, etoposide, 5-fluorouracil, hydroxyurea, streptozocin and 6-mercaptopurine could be effectively combined with adjuvant NK based immunotherapy without affecting the killing of NK cells.
4. Our findings point to a potential hazard of giving G-CSF to ALL patients with febrile neutropenia, especially those which coexpress myeloid markers and G-CSFR and treated with daunorubicin. On the other hand adjuvant treatment of AML patients with G-CSF in order to prevent neutropenia, or its use in priming regimens might result resistance to daunorubicin.

Summary

The survival of patients with malignant diseases can be increased in the future using more individualized therapies. Malignancies are more heterogenous in their drug sensitivity than they are rated into different subgroups for cytostatic treatment. Using of effective drugs determined by in vitro drug sensitivity test might result in a better clinical outcome. The most frequently used chemotherapy regimes originate from the non-Hodgkin lymphoma protocols and there are no specific cytotoxic drugs that would have been specifically selected against EBV induced lymphoproliferative disorders. As lymphoblastoid cell lines (LCLs) are well established *in vitro* models for posttransplant lymphoproliferative disorders (PTLD), we have assessed 17 LCLs for cytotoxic drug sensitivity. The precise number of living and dead cells was determined using a custom made automated laser confocal fluorescent microscope. Independently from their origin, LCLs showed very similar drug sensitivity patterns against 29 frequently used cytostatic drugs. LCLs were highly sensitive for vincristine, methotrexate, epirubicin and paclitaxel. Our data suggest that the inclusion of epirubicin and paclitaxel into chemotherapy protocols against PTLD may be justified. We characterized the effect of 28 frequently used chemotherapeutic agents on the capacity of NK cells to kill target cells. We found that treatment of NK cells with the drugs:including vinblastine, paclitaxel, docetaxel, cladribine, chlorambucil, bortezomib, MG-132 effectively inhibited NK mediated killing, without affecting the viability of NK cells. On the other hand we found drugs that permitted efficient NK-mediated killing even at concentrations comparable to or higher than the maximally achieved therapeutic concentration *in vivo*, in humans. We suggest that these drugs which include asparaginase, bevacizumab, bleomycin, doxorubicin, epirubicine, etoposide, 5-fluorouracil, hydroxyurea, streptozocin and 6-mercaptopurine could be combined effectivelly with NK based adjuvant immunotherapy:. Using MTT assay we also investigated how G-CSF might influence the sensitivity of leukemic cells to daunorubicin induced cell death.. After pretreatment of KG-1 leukaemic cells with G-CSF a moderate increase in the resistance of the cells to daunorubicin could be observed. This may draw attention to the risk of G-CSF application as an adjuvant therapy of AML and childhood ALL. In summary, we show in this thesis possibble ways to use the in vitro drug sensitivity assay for the clinical practice. In case of some patients where no effective protocols by proved multicentric randomized studies are available, as well as in case of rare malignant diseases or in advanced tumors, our new in vitro drug sensitivity assay may lead to develop novel cytostatic protocols against these malignancies.

Publication list

Publications

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