INTRODUCTION

Apoptosis is a physiological cell death program that occurs in all eukaryotic organisms. Its two main purposes for the organism are to allow tissue remodeling, especially during embryogenesis, and to induce cell death in damaged cells in order to prevent genetic instability. Damage of the apoptotic program can lead to tumor formation by shifting the tightly regulated balance between proliferation and apoptosis into the direction of cell growth, and can result in drug resistance, since radiotherapy and most chemotherapeutic agents induce cell death via apoptosis. Knowledge of the apoptotic pathways, therefore, is of utmost importance for the understanding of tumor pathogenesis and of drug resistance.

Apoptosis is an active process in which the caspases, a family of cysteine-proteases are the key mediators. In humans, 14 different caspases are known. They are synthesized as inactive pro-enzymes consisting of a pro-domain, a large and small subunit. The active caspases are tetramers composed of two large and two small subunits arising from the cleavage of two pro-caspases. So-called initiator caspases, such as caspases-8, -9 and -10 receive the apoptotic signal, get activated and activate themselves so-called executioner caspases such as caspase-3, -6 and -7. These induce the apoptotic phenotype by cleavage of enzymes such as the DNA-repair-enzyme PARP, activation of endonucleases and cleavage of structural proteins such as the lamins, which are important for the maintenance of the nuclear membrane. The activity of the caspases is tightly controlled by proteins of the “inhibitor of apoptosis family” (IAPs), to which the proteins XIAP, c-IAP1, c-IAP2, NAIP, survivin, livin, Ts-IAP, and BRUCE belong.

There are two major apoptotic pathways, the intrinsic and extrinsic pathway. The intrinsic apoptotic pathway is activated when cells are critically damaged. A key event in the intrinsic pathway is the permeabilization of the mitochondrial membrane leading to the release of cytochrome c which binds the cytosolic protein Apaf 1 (apoptosis activating factor 1). Both proteins together bind pro-caspase-9 and form the apoptosome, where pro-casapase-9 gets cleaved into active caspase-9. In addition, permeabilization of the mitochondria leads to the release of proteins SMAC/DIABLO and HtrA2/Omi, which both bind to and inactivate proteins of the IAP-family. The permeability of the mitochondrial membrane is controlled by a tight balance between pro- and anti-apoptotic members of the BCL2 family of proteins. The pro-apoptotic members comprise proteins such as Bax, Bak, and Bad, which contain three Bcl-2 homology domains (BH1-3) and proteins with only one domain (BH-3) such as Bid, Bim, PUMA, and Noxa. The anti-apoptotic members are Bcl-2, Bcl-XL, Bcl-w and Mcl-1. Bcl-2 and Bcl-XL form stable complexes with the pro-apoptotic BH-3 domain only proteins, thereby preventing the activation and translocation of Bax and Bak to the mitochondria. This balance of pro- and anti-apoptotic members of the BCL2 family of proteins is influenced by p53 which gets activated upon cellular stress. P53 induces transcriptional activation of the pro-apoptotic Bax and PUMA, thereby shifting the balanced network of BCL2 family proteins towards apoptosis. Since most chemotherapeutic agents induce intracellular damage, they induce apoptosis via the intrinsic pathway. Disruption of the intrinsic pathway therefore often leads to chemoresistance. The causes can be manifold such as mutations in pro-apoptotic genes, e.g. p53, or Bax, or overexpression of anti-apoptotic proteins, e.g. Bcl-2 or survivin.
Activation of cell surface receptors belonging to the death receptor family initiates the extrinsic apoptotic pathway. This family comprises TNF-R1, Fas (also called CD95 or APO-1), DR3, TRAIL-receptors 1 and 2 (also called DR4 and DR5), and DR6. Binding of their respective ligand leads to trimerization of the death receptors, resulting in a conformational change of the intracellular “death domain”, and allowing its binding to an adaptor molecule which is FADD for Fas, TRAIL-R1 and -R2, TRADD for TNF-R1 and an unknown complex – involving TRADD and Bax – interacting with the cytoplasmic tail of DR6. The bound adaptor molecules recruit and activate pro-caspases-8 and -10. The complex of death receptor, adaptor protein and initiator caspase is referred to as death inducing signaling complex (DISC). Caspases-8 and -10 activate effector caspases and the pro-apoptotic BCL2 family protein Bid. In cells, in which activation of death receptors generates a high intracellular concentration of active caspase-8 and/or -10, sufficient activation of effector caspases leading to apoptosis ensues (type I-cells). In cells with little generation of caspase-8 and/or -10, activation of the intrinsic pathway via Bid is necessary for the induction of apoptosis (type II-cells). As in the intrinsic pathway activation of p53 modulates the extrinsic pathway towards apoptosis. Activated p53 results in transactivation of the death receptors Fas and TRAIL-R2.

Apoptosis via the extrinsic pathway is tightly controlled and resistance can occur at various levels. On the receptor level there are TRAIL-R3 and -R4, which do not contain a death domain and can sequester TRAIL from the apoptosis inducing receptors TRAIL-R1 and -R2. Overexpression of the protein cFLIP has been shown to prevent the binding of the adaptor FADD to pro-caspase-8 and -10. Mutations in Fas as well as absent expression of death receptors or caspases-8 and -10 are also known causes of resistance to the induction of apoptosis via the extrinsic pathway.

In addition to apoptotic signaling survival and proliferation can be induced in particular by TNFR activation. The intracytoplasmic tail of the receptor itself and the adaptor protein TRADD can interact with TRAF2 (TNF receptor-associated factor 2), a strong activator of NFkB and AP1. The change of gene expression can promote cell survival and proliferation.

Another response to cellular stress besides apoptosis is autophagy. Instead of committing suicide cells, proteins and whole cell organelles are engulfed in double membrane vesicles – the autophagosome. Fusion of the autophagosome with a lysosome and subsequent degradation of proteins and lipids to basic biomolecules serve as a basis for recycling in times of starvation, to degrade harmful molecules, and intracellular pathogens as well as keeping cellular homeostasis. Autophagy can precede apoptosis and may serve as an alternative mechanism to activate caspase-8. Members of the BCL2 family are involved in regulation of autophagy as well. One example is the interaction of Bcl-2 with Beclin 1, the central regulator of autophagy. Bcl-2 forms a complex with Beclin 1 inhibiting autophagy. Dissociation of this complex initiates autophagy.

Autophagy might contribute to resistance of tumor cells towards environmental stress factors like nutrient deprivation and hypoxia. Inhibition of autophagy in this context can lead to induction of apoptosis. On the other hand autophagy itself might serve as an alternative pathway to induce cell death known as “autophagic cell death”. In particular, in tumor cells with defective apoptotic pathways, massive degradation of intracellular components leads to cell death. Drugs inducing autophagic cell death might therefore complement apoptosis-inducing anti-cancer agents in order to maximize the cytotoxic effect on a tumor.

In the following, the apoptotic pathways in malignant bone tumors are described in detail. Insight into which pathways are intact and how deficient pathways can be restored could be of potential value for the design of new treatment concepts.

OSTEOSARCOMA

The Fas/Fas ligand pathway

Fas surface expression is found in the majority of osteosarcoma (OS) cell lines. Hamada and coworkers detected Fas surface expression in 10 out of 14 OS cell lines examined; in the remaining four cell lines Fas was only detected in the cytoplasm. Only two out of 14 cell lines were susceptible to apoptosis by anti-Fas antibody. Coincubation with the protein-synthesis inhibitor cycloheximide induced Fas-mediated apoptosis in 10 cell lines. Similar results were observed by Fellenberg and colleagues. Whereas all three OS cell lines had surface expression of Fas, anti-Fas antibody alone was not able to induce apoptosis. Coincubation with cycloheximide or incubation with TNF rendered OS cells sensitive to Fas-mediated apoptosis. Interestingly, OS cell lines produced a soluble form of Fas which was able to protect Jurkat cells from anti-Fas antibody-mediated apoptosis. Inaba and colleagues analyzed the effect of interferon-gamma on Fas-mediated apoptosis in four OS cell lines. Interferon-gamma increased Fas- and caspase-8 expression in all four cell lines. Whereas interferon-gamma or anti-Fas-antibody alone only caused minimal apoptosis, the combination of both substances resulted in marked apoptosis.

II. PRIMARY BONE TUMORS
Loss of Fas expression has been linked to tumor progression in various cancers. Kleinerman and coworkers have been investigating the role of the Fas-signaling pathway on the development of metastases in osteosarcoma. By selection of variants of the OS cell line Saos with different degrees of Fas expression as well as by transfection of Fas, they were able to show that the degree of Fas expression inversely correlated with the metastatic potential of the cell line variants in a mouse xenograft model. In addition, using a murine osteosarcoma model they could show that primary tumors expressed high levels of Fas, whereas no expression of Fas was present in actively growing lung metastases. They postulate that Fas-expressing OS cells are eliminated from the lungs through induction of apoptosis by Fas ligand expressing pulmonary epithelial cells. Similarly, when analyzing Fas expression in pulmonary metastases from patients with OS, they found that 60% of tumors did not express Fas and 32% only showed weak expression by immunohistochemistry. Whereas disruption of the Fas-pathway by transfection of K7M2 murine OS cells with a dominant-negative FADD mutant resulted in a higher number of pulmonary metastases, inhalation of gemcitabine by mice upregulated Fas expression of pulmonary nodules and reduced tumor growth. Recent data has shown that miRNAs are involved in the regulation of Fas expression in osteosarcoma metastasis. miR-20a was highly expressed in cells with low Fas expression. Furthermore inhibiting expression of miR-20a in LM7 cells, a subline of Saos, expressing low levels of Fas, led to reduction of pulmonary metastasis in a mouse xenograft model. Also, Fas expression in OS cells was shown to be upregulated by IL-12. Intranasal gene therapy with the IL-12 gene of Saos-bearing mice xenografts and treatment with ifosfamide, which upregulates the expression of Fas ligand in OS cells, inhibited the formation of lung metastases.

Another possibility to modulate the Fas pathway is altering expression of cFLIP. Rao-Bindal and coworkers showed that the histone deacetylase inhibitor MS-275 reduced cFLIP expression in pulmonary metastasis in mice and led to marked apoptosis and tumor regression. On the other hand, even in primary tumors expressing low levels of Fas, tumor regression through the application of FasL delivered intratumoral via gene therapy in dog osteosarcoma has been observed. The gene therapy led to an inflammatory anti-tumor response. Surprisingly, EFS and OS in dogs bearing tumors with low level Fas expression was better compared to dogs with tumors expressing high levels of Fas. The authors postulate that Fas-expressing neutrophils surrounding necrotic as well as fibrotic tumor areas are killed by FasL and the subsequent induction of inflammation serves as a basis for a robust anti-tumor immune response.

The TRAIL pathway

In primary osteosarcomas and osteosarcoma cell lines sequence variations in the TRAIL-R1 gene but not TRAIL-R2 or FADD gene have been described. Since these sequence variations are homozygous in 15% of tumor samples and cell lines but not in control cells, it is suggested that they influence ligand-receptor interactions and subsequent apoptosis induction. TRAIL-signaling in osteosarcoma has been studied by various groups in vitro. In contrast to ES cells, only a minority of OS cell lines are sensitive to TRAIL-mediated apoptosis. Incubation of resistant cell lines with cytotoxic drugs doxorubicin, cisplatin and etoposide but not methotrexate or cyclophosphamide could sensitize them to TRAIL-mediated apoptosis. No effect of TRAIL alone or in combination with cytotoxic drugs could be observed in human osteoblasts. The sensitizing effect of doxorubicin and cisplatin to TRAIL in osteosarcoma cells has been further analyzed in U2OS cells and was shown to rely on the downregulation of the inhibitory apoptotic protein XIAP, an inhibitor of caspases-3 and -9. Whereas the execution of apoptosis in OS cells by TRAIL is caspase-dependent, apoptosis induction has been shown not to be dependent on FADD and caspase-8 in U2OS cells. Inhibition of the two proteins with siRNA did not protect from TRAIL-mediated apoptosis. In contrast, inhibition of Bid or cathepsin B, which has been shown to mediate TRAIL-induced apoptosis in oral cancer cells, prevented apoptosis by TRAIL. In cell line BTK-143, however, TRAIL-mediated apoptosis could be prevented by a transfection of a dominant-negative form of FADD. This suggests diversity in the TRAIL-signaling pathway, even in one tumor entity. Resistance to TRAIL was acquired in vitro by BTK-143 cells through expression of TRAIL-R4. This receptor lacks a functional death domain and cannot mediate apoptosis. Sensitivity against TRAIL could be restored by blocking of TRAIL-R4 with a specific antibody and by incubation of cells with doxorubicin, etoposide and cisplatin. Cenni and coworkers analyzed the TRAIL-sensitivity of OS cell line U2OS and a MDR-expressing subline. Both clones expressed TRAIL-receptors to a similar amount. While the parent clone was TRAIL-resistant, the MDR-expression clone was TRAIL-sensitive. Analysis of post-receptor events revealed that TRAIL-responsiveness inversely correlated with activation of Akt. Expression of a constitutively active Akt in MDR-U2OS decreased TRAIL-sensitivity.

Evaluating targeted therapy with lexatumumab, a human monoclonal antibody against TRAIL-R2, in 24 pediatric patients with sarcomas, among them nine patients with osteosarcoma, did not show a complete or partial response in any case. However, a patient with chest wall osteosarcoma included in the study 4 weeks after radiation therapy had
negative PET-scans and a sustained improvement of clinical status after lexatumumab therapy. In general, patients who underwent radiotherapy in front of lexatumumab therapy seemed to have better tumor response rates probably due to upregulation of TRAIL-R2 by frontline radiotherapy. Dose limiting toxicity, i.e. pleural effusion and hypoxia was observed in one patient with pleural-based synovial sarcoma. Particularly, no grade 3 or 4 liver toxicity has been observed28.

Drug-induced apoptosis

Fellenberg and coworkers demonstrated that chemotherapeutic drugs doxorubicin, methotrexate and cisplatin induced apoptosis in three different OS cell lines (HOS/TE-85, MG63, Saos-2)13. Induction of apoptosis resulted in the reduction of the mitochondrial potential and cytochrome c release into the cytoplasm and was caspase-dependent. No increase in the expression of Fas or induction of FasL-expression by chemotherapeutics was observed. However, all four cell lines were either p53 null or expressed mutant p53, while Fas expression has been shown to be upregulated by wt p53 in various cell systems. Also, no inhibition of drug-induced apoptosis was seen when anti-FasL antibody was added.

Paclitaxel

Paclitaxel induces cytotoxicity through inhibition of microtubuli formation. In the OS cell line U2OS paclitaxel induces G2/M arrest and apoptosis via the activation of caspase-3. Also, caspase-3 mRNA expression is increased after incubation of cells with paclitaxel29. In the Saos-2 cell line paclitaxel induced apoptosis through the mitochondrial pathway and cytochrome c release. In a fraction of treated cells paclitaxel induced autophagy, thereby preventing apoptotic cell death. Inhibition of autophagy promoted paclitaxel-induced apoptosis via an increase of cyctochrome c release and caspase-3 activation30.

Histone deacetylase inhibitors

Histone acetylation is regulated by the balance of histone acetyltransferase (HAT) and histone deacetylase (HDAC) and plays an important role in regulating gene expression by modulating chromatine structure. HDAC inhibitors can cause a variety of biological effects such as cell cycle arrest, apoptosis and differentiation. There are seven structurally distinct classes of inhibitors. Their biochemical matrix includes hydroxamic acids, cyclic peptides, short-chain fatty acids, and benzamides31. In osteosarcoma cells the HDAC inhibitor FR901228 has been shown to downregulate cFLIP by inhibiting generation of cFLIP mRNA, thereby sensitizing Fas-resistant OS cell lines to Fas-mediated apoptosis32. In another study, valproic acid was found to decrease the secretion of soluble Fas by OS cell lines and to sensitize cell lines against Fas-mediated apoptosis33. The combination of valproic acid and hydralazine, a DNA methylation agent, increased cell surface expression of Fas and Fas-mediated apoptosis. Furthermore, valproic acid enhanced the expression of MHC class I-related chains alpha and beta increasing NK cell-mediated cytotoxicity in U2OS, Saos-2 and HOS cell lines in vitro34. HDAC inhibitors also sensitize tumor cells towards TRAIL-induced apoptosis. In OS cell lines Saos-2 and DAOY, the combination of TRAIL and vorinostat led to apoptosis through increased activation of caspase-835. Synergistic effects on apoptotic cell death were also shown for a combination of the histone deacetylase inhibitor PCI-24781 and doxorubicin in the cell line U2OS36.

Whereas HDAC inhibitors are only cytotoxic to tumor cells at doses usually not tolerated in vivo, lower dosages administered over a prolonged time of 7 days have been shown to induce differentiation of osteosarcoma cells. Cain and coworkers demonstrated that lower doses of the histone deacetylase inhibitor LBH589 induced differentiation of OS cells into chondrocytes, adipocytes or osteoblasts in vitro. Using a mouse xenograft model they were able to show that LBH589 reduced tumor growth and induced a differentiation program in tumor cells in vivo. Gene expression analysis revealed additional upregulation of mRNA of genes involved in cell cycle arrest37.

Proteasome inhibitors

The ubiquitine-proteasome system plays a major role in cell proliferation and cell death. Its inhibition by proteasome inhibitors offers a new therapeutic strategy for cancer treatment. The proteasome inhibitor MG132 induced apoptosis in OS cell line Saos-2 via an increase in level of reactive oxygen species (ROS), mitochondrial membrane depolarization with release of cytochrome c and subsequent caspase activation. Introduction of the RB gene into Saos-2 cells had a protective influence, possibly due to increased levels of Bcl-2, whereas introduction of p53 potentiated the apoptotic effect of MG13238.

Bortezomib treatment of OS cell lines HOS, 143B and OS187 in vitro inhibited degradation of the osteoblastic differentiation marker and inducer of Bax expression Runx2. Treatment with bortezomib in an osteosarcoma mouse xenograft model using cell line 143B demonstrated growth arrest and induction of apoptosis in tumor cells39.
**Flavopiridol**

Flavopiridol is a pan-cyclin-dependent kinase (CDK) inhibitor that induces cell cycle arrest and apoptosis in many cancer cells. Flavopiridol induced apoptosis in ES cell line WE-68 and OS cell line MNNG. Apoptosis was also observed in P-glycoprotein and multidrug resistance-associated protein 1 overexpressing subclones which were resistant to doxorubicin. Flavopiridol caused release of mitochondrial cytochrome c and activation of caspases-9, -3 and -8. Apoptosis was inhibited by pan-caspase and caspase-3 inhibitor, but not caspase-8 inhibitor, suggesting activation of the intrinsic pathway.

**Zoledronic acid**

Zoledronic acid (ZOL) is an amino-bisphosphonate (N-BP) that inhibits osteoclast-mediated bone resorption. In addition, ZOL inhibits proliferation and induces apoptosis in various tumor cell lines. In OS cells, ZOL has been shown to activate an intra-S DNA checkpoint with an increase in P-ATR, P-chk1, Wee 1, and P-cdc2 and a decrease in cdc25, independent of the p53 and Rb status. Induction of apoptosis has been demonstrated to be caspase-independent and to be characterized by increased mitochondrial permeability with translocation of apoptosis-inducing factor (AIF) and endonuclease G from a mitochondrial to a perinuclear location. In an orthotopic OS mouse xenograft model, ZOL resulted in inhibition of primary tumor growth and reduction of lung metastases of Saos-2 tumor bearing mice. ZOL also inhibited the formation of lung metastases in a murine osteosarcoma model using the cell line POS-1.

Combination of ZOL and TRAIL was able to sensitize primary TRAIL-resistant MG63 cells to apoptosis. Coincubation of MG63 cells with alendronate, another N-BP, and TRAIL increased expression of TRAIL-R2 in vitro. In addition, coincubation of alendronate with TRAIL enhanced the inhibition of protein prenylation, a process interacting with protein trafficking, compared to incubation with alendronate alone. This indicates that inhibition of protein prenylation contributes to sensitization towards TRAIL-induced apoptosis.

ZOL also sensitizes OS cells to be eliminated by immune effector cells. The effect relies on the feature of ZOL to induce proliferation of γδT cells, enhance their cytolytic properties and sensitize tumor cells to cytotoxic killing by γδT cells. OS cell lines pretreated with ZOL were efficiently killed in vitro by Vγ9Vδ2 T cells via T-cell receptor-mediated recognition through the perforin and partly TRAIL pathways.

**Statins**

Statins are cholesterol-lowering agents which recently have been found to trigger cell death in a variety of tumor cells. Statins inhibit the 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, which catalyzes the conversion of HMG-CoA into mevalonate during cholesterol biosynthesis. Blocking this pathway by statins produces similar effects to those of bisphosphonates, with decreased prenylation of proteins, such as small G proteins, altering cell growth and survival. Fromigue and coworkers demonstrated that lipophilic statins induced apoptosis in OS cell lines Saos-2 and OHS4 by inactivation of the small G protein RhoA. Inactivation of RhoA resulted in decreased levels of p42/p44-MAPKs, Bcl-2 and Mcl-1.

**Inhibition of glutathione S-transferase P1 (GSTP)**

GSTP is one of the cytosolic glutathione transferases and detoxifies a wide variety of electrophilic compounds including certain anti-cancer agents. Overexpression of GSTP1 has been linked to chemoresistance in various cancers. When establishing a series of cisplatin-resistant OS cell lines, Pasello and coworkers found that resistance to cisplatin was mainly associated with an increase of both the intracellular level and enzymatic activity of GSTP1. Overexpression of GSTP1 in OS cells caused cells to be more resistant to doxorubicin and cisplatin, whereas GSTP1 suppression by siRNA resulted in growth inhibition and an increase in apoptosis to both drugs. Treatment of cisplatin-resistant OS cell lines with the GSTP1-inhibitor 6-(7-nitro-2,1,3-benozoxydiazol-4ylthio)hexanol (NBDHEX) sensitized cells to cisplatin cytotoxicity in vitro through disruption of the GSTP1:TRAF2 complex. TRAF2 (TNF receptor-associated protein 2) binds to ASK and leads to activation of JNK and p38 thereby inducing cell cycle arrest and apoptosis. Analyzing 34 patients with high-grade osteosarcoma, increased expression of GSTP1 was associated with a significantly higher relapse rate and worse clinical outcome. Furthermore, OS primary tumor cells express different forms of GSTP1 and response to neoadjuvant chemotherapy can depend on the expressed polymorphism of GSTP1. In a study by Yang and coworkers patients with tumors expressing the Val/Val genotype had significantly better response rates to chemotherapy compared to patients with tumors expressing the Ile/Ile genotype.

**Apoptosis-relevant genes as prognostic markers**

Mutations in the tumor suppressor gene *p53* are found in 20–60% of patients with sporadic osteosarcoma. Whereas *p53* mutations are associated with an aggressive tumor phenotype, chemoresistance and poor outcome in
a variety of malignancies, a large prospective study did not find an influence of p53-mutations on chemoresistance and clinical outcome in osteosarcoma. In a smaller study, 35 primary osteosarcoma specimens were analyzed for the expression of p53, Bax and Bcl-2 by immunohistochemistry. None of the parameters studied correlated with prognosis. However, patients who were Bax + /Bcl-2-/p53+ had significantly lower 4-year disease-free survival than other patients. Expression of Bcl-X, mRNA, another anti-apoptotic BCL2 family member, is significantly increased in tumors of patients with advanced clinical stage and hematogenous metastases. Patients with tumors expressing high levels of Bcl-X, had a higher incidence of hematogenous metastases at diagnosis and decreased overall survival. Functional studies in U2OS and MG63 cells revealed miR-133a as a negative regulator of Bcl-X expression. Restoration of miR-133a led to increased apoptosis in cells treated with serum deprivation and hypoxia. In vivo transfected cell lines showed dramatic reduction in tumor size and delayed tumor formation in mice. This correlates to the decreased expression of miR-133a in tumors of patients with a poor clinical course and reduced overall survival.

Survivin is a member of the inhibitor of apoptosis protein (IAP) family. It inhibits apoptosis by binding directly to both caspases-3 and -7. When analyzing the initial biopsy specimen of 22 patients with OS, Osaka and coworkers could demonstrate that patients with metastases had significantly higher mRNA levels of survivin than patients without metastases, and that the 5-year survival of patients with high survivin levels was significantly lower than the one for patients with low survivin levels.

**APOPTOSIS IN EWING SARCOMA**

**The Fas/Fas ligand pathway**

Expression of FasL by immunohistochemistry has been reported in 63.5% and Fas in 79.4% of primary Ewing sarcoma (ES). A higher expression of FasL (95%) has been found in metastatic tumors suggesting an association with a metastatic phenotype. In ES cell lines, apoptosis through the Fas signaling pathway could only be induced in 30% of Fas-expressing cell lines. When cells were treated with a combination of an anti-Fas antibody and the protein synthesis inhibitor cycloheximide, seven out of nine cell lines became Fas-sensitive. As in primary cells, simultaneous expression of Fas and FasL in cell lines was observed, even in Fas-sensitive cells. Expression of FasL was strictly confined to the cytoplasm in one report, whereas also transmembrane expression and secretion of soluble FasL by metalloproteinase into the supernatant was observed by another group. Inhibition of FasL shedding by matrix metalloproteinase inhibitors resulted in Fas-mediated apoptosis in Fas-sensitive ES cell lines.

Zhou and colleagues demonstrated that transfection of ES cell line TC71 with an adenovirus carrying cDNA of the IL-12 gene upregulated Fas-expression. Whether transfected cells became sensitive to Fas-mediated apoptosis, however, has not been shown. Intratumoral injection of adenovirus murine IL-12 into mice bearing xenografts of ES cell line TC71 inhibited tumor growth of primary tumor as well as growth of an untreated tumor on the contralateral side. Immunohistochemistry of treated tumors demonstrated increased expression of Fas, FasL as well as tumor cell apoptosis.

Melatonin was shown to induce apoptosis in the ES cell lines SK-N-MC, A673, A4573, TC71 and SK-ES1 by increasing the expression of Fas and FasL on the cell surface. Apoptosis could be blocked by incubation of SK-N-MC cells with z-IETD-FMK, a caspase-8 inhibitor.

**The TRAIL pathway**

Ewing sarcoma cells do express receptors for TRAIL. Mitsiades and coworkers found that 94% of tumors from patients with ES expressed TRAIL-R1 and 75% TRAIL-R2 by immunohistochemistry. Either one of the TRAIL-receptors was expressed by 97%. In contrast to the Fas signaling pathway, the TRAIL-pathway seems to be intact in the majority of ES cells and its activation leads to apoptosis in about 80% of ES cell lines examined. Resistance to TRAIL in ES cell lines has been linked to deficient expression of caspase-8. Heterogeneous expression of caspase-8, however, has also been observed in tumors from patients with ES and therefore could be a major obstacle to the success of TRAIL receptor-targeted therapy. We have shown that 25% of tumors from patients with ES had less than 50% of tumor cells expressing caspase-8. As in neuroblastoma, the lack of expression of caspase-8 is due to methylation within the caspase-8 gene. A means to re-express caspase-8 in ES tumor cells could be the application of interferon-gamma. We were able to show that interferon-gamma in concentration of 10–20 U/ml leads to re-expression of caspase-8 in ES cell lines and sensitizes cell lines to TRAIL-mediated apoptosis. Since in humans interferon-gamma concentrations of up to about 80 U/ml can be achieved, the therapeutic application of interferon-gamma in patients...
with ES could increase caspase-8 expression in tumors and make them amenable to therapy with TRAIL receptor agonists. TRAIL is also able to suppress the growth of Ewing tumors in an orthotopic ES xenograft model in which tumor cells were injected into the gastrocnemius muscle. Interestingly, when the tumor bearing extremities were amputated and mice were followed up for the development of metastases, mice initially treated with TRAIL or vehicle died within 2 months of metastases. In contrast, treatment with interferon-gamma alone or in combination with TRAIL did significantly decrease the incidence of metastatic disease with 60% of the mice treated with TRAIL and interferon-gamma still being alive at the end of the 6-month observation period.

Another mechanism of resistance to apoptosis by TRAIL is downregulation of TRAIL-R1 expression. Picarda and colleagues showed that downregulation of TRAIL-R1 expression in the TRAIL-sensitive cell line TC71 leads to inhibition of apoptosis even in presence of TRAIL-R2 expression. Furthermore, they characterized a short isoform of TRAIL-R1, the bDR4 receptor, whose expression is linked to TRAIL sensitivity.

In another in vivo study by Picarda and coworkers TRAIL has been administered via non-viral gene therapy by a third generation phosphonolipid transfection reagent. Treated mice showed a lower tumor incidence, reduced tumor growth and better survival with a lower metastasis rate than controls.

The sensitivity of ES cells to TRAIL can be influenced by IGF1. Van Valen and colleagues showed that short time incubation of VH-64 cells with IGF1 for 24 h leads to decreased sensitivity to TRAIL-mediated apoptosis. In contrast, longer incubation times up to 72 h resulted in sensitization towards TRAIL-induced apoptosis. Differential levels of expression of XIAP, dependent on the duration of incubation with IGF1, were shown to be responsible for this effect. There was no effect in primary TRAIL-resistant cell lines. Apoptotic sensitivity towards doxorubicin and etoposide was reduced by long-term IGF1 treatment.

**CD99-induced apoptosis**

The CD99 antigen is consistently expressed in ES and therefore used as a marker to distinguish ES from other small blue round cell tumors. Its expression is regulated by EWS-FLI1-mediated inhibition of miR-30a-5p. CD99 has been shown to be involved in the transendothelial migration of human neutrophils. It is expressed in cells of all leukocyte lineages. The degree of expression is high in immature bone marrow precursors, diminishes with differentiation of cells and is low in peripheral blood cells. Since CD99 is highly and consistently expressed in ES, it represents an attractive target for therapeutic intervention. Ligation of CD99 with a specific antibody leads to rapid aggregation of cells and caspase-independent apoptosis. Apoptosis can be inhibited by silencing of the zyxin gene, which codes a protein involved in the regulation of the actin cytoskeleton whose expression is upregulated after CD99 engagement. Simultaneous administration of an anti-CD99 antibody with doxorubicin had a synergistic effect on inhibition of cell growth in vitro. In athymic mice, the combination of anti-CD99 and doxorubicin proved to be more active in inhibiting the growth of primary tumors as well as reducing the number of lung and bone metastases than each component alone.

**Drug-induced apoptosis**

A role for the Fas/FasL-pathway in doxorubicin-mediated cytotoxicity has been shown by Mitsiades. Doxorubicin-mediated apoptosis was reduced in cells of the ES cell line SK-N-MC either by a soluble form of Fas, acting as a decoy inhibitor of membrane-bound Fas or by matrix metalloproteinase-7 through cleavage of membrane-bound FasL. However, no role for caspase-8 in doxorubicin or etoposide-induced apoptosis has been demonstrated in ES cell lines A4573 and JR. Neither cell line expresses initiator caspases-8 and -10, required for death receptor-mediated apoptosis. Transfection of wt caspase-8 or induction of caspase-8 expression by interferon-gamma did not alter their sensitivity to drug-mediated apoptosis. In patients with ES, caspase-8-expression of primary tumors did not correlate with event-free or overall survival.

Resistance towards doxorubicin-induced apoptosis in vitro can be attributed to overexpression of miR-125b leading to downregulation of p53 and Bak.

**Treosulfan**

Treosulfan is a bifunctional alkylating agent which is structurally related to busulfan. In contrast to busulfan, treosulfan causes fewer adverse effects in patients, even allowing high-dose chemotherapy in previously irradiated patients. Treosulfan has been shown to be more active against ES xenografts than busulfan in a mouse xenograft-model. Apoptosis is induced via the intrinsic pathway and involves caspase-9 and the effector-caspase-3.
Fenretinide

Fenretinide is a synthetic vitamin A analog. It induces apoptosis in ES cell lines at concentrations achievable in vivo and slows the growth of ES xenograft in nude mice[82,83]. Myatt and coworkers investigated the induction of apoptosis by fenretinide in ES cells. Fenretinide led to the accumulation of reactive oxygen species (ROS) within 5 min. This was followed by the activation of p38MAPK. An increase in the mitochondrial membrane permeability and release of cytochrome c was observed after 8 h and dependent on ROS and the activation of p38MAPK[83]. Activation of p38MAPK leads to upregulation of death receptor expression, including TRAIL-R1 and -R2, Fas and p75 neurotrophine. Combined treatment of cells with fenretinide and TRAIL had a synergistic effect on apoptosis[84].

Zoledronic acid

Zoledronic acid (ZOL) has been shown to induce apoptosis in ES cell line TC71. Apoptosis was increased in a synergistic way with paclitaxel[85]. In a mouse xenograft model, in which TC71 tumor cells were injected intratibially, ZOL alone inhibited the development of bone tumors. Whereas paclitaxel alone had no inhibiting effect on the development of primary tumors, the combination therapy of ZOL and paclitaxel led to tumors in only 22% of mice versus 89% in the control group.

In contrast, Odri and colleagues showed that ZOL alone did not have an effect on tumor growth of A673 or TC71 tumor cells in soft tissues in a mouse xenograft model. Nevertheless the authors could show that the administration of one cycle of ZOL in combination with ifosfamide had a greater growth inhibitory effect than three cycles of ifosfamide alone. Focusing on bone ES, the results by Zhou and coworkers could be confirmed. ZOL inhibited development of primary tumors and showed a synergistic effect on tumor growth in mice. Furthermore tumor relapse after ifosfamide monotherapy could be prevented by ZOL. The bone architecture was conserved in the combinational group[86].

Another N-BP, minodronate, was shown to inhibit ERK and AKT phosphorylation in SK-ES-1 ES cell lines. Combination of doxorubicin and minodronate led to reduced tumor growth in vitro and in a mouse xenograft model[87].

Proteasome inhibitors

ES cell lines were found to be highly sensitive to the proteasome inhibitor bortezomib in vitro. Bortezomib was able to induce apoptosis via activation of caspase-3, cleavage of PARP and induction of p21 and p27. In addition, it exhibited synergistic activity with TRAIL in some ES cell lines[88].

PARP inhibitors

When screening a panel of several hundred human tumor cell lines against 130 drugs used clinically and pre-clinically, Garnett and coworkers discovered that ES cell lines were more sensitive towards induction of apoptosis by PARP inhibitors than cell lines from other tumors[89]. The enzyme PARP plays a major role in base excision repair and is cleaved by executioner caspases during apoptosis. The marked sensitivity of ES cells against PARP inhibitors was shown to be due to an increase of EWS-FLI1-induced DNA damage by inhibition of PARP1[90]. Combination of the alkylating agent temozolamide with the PARP inhibitor olaparib resulted in complete remission of Ewing tumors in a mouse xenograft model.

Aurora kinase inhibitors

Aurora kinases are closely linked to regulation of cell division and organization of mitosis. Three kinases, Aurora-A, -B and -C, have been described in mammals. In particular Aurora-A and -B are involved in organization of the centrosome and chromosomal alignment. In absence of p53, alteration of mitotic organization by overexpression of Aurora kinases leads to aneuploidy and transformation. Inhibiting Aurora-Kinase function via RNAi or kinase inhibitors led to growth inhibition and apoptosis in vitro and in vivo in a great variety of tumor cells[91,92].

Employing a focused screen with 200 small molecule protein kinase inhibitors in different ES cell lines revealed the Aurora kinase inhibitors tozasertib and danusertib as most potent inhibitors of cell viability. Though interacting with over 20 target kinases tozasertib was shown to most potently inhibit Aurora kinases. Inhibition of their expression via RNAi induced similar reduction of cell viability compared to Aurora kinase inhibitor treatment in vitro. Cells treated with tozasertib underwent apoptotic cell death showing activation of caspase-3. Furthermore synergistic action of tozasertib in combination with doxorubicin and etoposide as well as reduction of tumor growth in a ES mouse xenograft model could be observed[93].

Apoptosis-relevant genes as prognostic markers

Aberrations in p53 have been found in about 10% of tumor samples from patients with ES either by aberrant expression detected by immunohistochemistry or by molecular methods[94-97]. In contrast, the majority of ES cell lines
contain alterations of p53, indicating selective pressure in the process of establishing in vitro growth. p53 overexpression has been associated with a significantly poorer survival in several studies. Ewing tumors containing either p53 or p16/p14ARF alterations showed a poor histological response to chemotherapy. When wild-type p53 was reintroduced by adenoviral transfection into cells of the ES cell line RH1, which contains mutp53, the transfected cells showed decreased viability and increased sensitivity to the chemotherapeutic agents cisplatin and doxorubicin.

Apoptosis in chondrosarcoma

Chondrosarcoma is characterized by high resistance towards chemotherapy and radiotherapy. Surgery comprises the most important treatment option. The expression of several proteins has been linked to chemotherapeutic resistance of chondrosarcoma cells in recent publications. Lechler and coworkers contribute resistance towards chemotherapeutic agents to high survivin expression in chondrosarcoma tumors. Inhibition of survivin expression sensitized SW1353 cells towards doxorubicin-induced apoptosis by increasing activation of caspase-3 and -7. Furthermore, expression of Bcl-2 and Bcl-XL was demonstrated to be involved in resistance to chemotherapy. Inhibition of both proteins by ABT-737, in combination with doxorubicin and cisplatin, resulted in a synergistic effect with regards to induction of apoptosis and reduction of cell viability in vitro. Combination therapy allowed for dose reduction of both chemotherapeutic drugs to sublethal concentrations. Apoptosis was accompanied by cytochrome c release. Kumari and colleagues identified LRF (leukemia-related factor) as another survival factor contributing to chemotherapeutic resistance. Expression of LRF was found in all three histopathologic grades of chondrosarcoma, but not in chondroma tissues. Inhibition of LRF expression in SW1353 and FS090 cells led to increased protein expression of p53 and p21, inhibition of cell proliferation, senescence, migration and invasion. Inhibition of LRF sensitized cells towards doxorubicin-induced apoptosis in vitro.

The chondrosarcoma cell line HTB-94 has been shown to be resistant to apoptosis via TRAIL. However, coinubcation with doxorubicin sensitized cells to TRAIL-mediated apoptosis. The mechanism of this has not yet been elucidated.

A synergistic action on the induction of apoptosis was found by combining cisplatin and rhPDCD5, a protein accelerating apoptosis in a variety of cancer cells. SW1353 cells treated with rhPDCD5 and cisplatin showed caspase-dependent induction of apoptosis in vitro and inhibition of tumor growth in a chondrosarcoma mouse xenograft model. Bcl-XL and Bcl-2 expression was found to be downregulated upon combined treatment whereas the expression of pro-apoptotic Bax increased.

Chondrosarcoma cells are relatively radiotherapy-resistant, requiring doses >60 Gy. Radiotherapy has been demonstrated to upregulate the expression of anti-apoptotic Bcl-2, Bcl-xL, and XIAP. When expression of the respective genes was inhibited using specific siRNAs, radiosensitivity increased markedly.

References


2. PRE-CLINICAL AND CLINICAL ASPECTS
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