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Meeting Report

# Differentiate or Die: the View from Montreal

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Over the last 15 years, the biennial conferences on Differentiation Therapy have sought to bring basic and clinical scientists together to stimulate discourse and establish collaborations to develop differentiation induction as a cancer therapy. The concept of differentiation therapy evolved from studies showing that cancer cells treated with certain chemicals or biologic peptides could frequently be induced to growth arrest and differentiate into the apparently normal counterparts of the tissues from which they were derived. The appreciation that induction of apoptosis was a developmental decision gone awry in cancer cells led to the recognition that an understanding of the signal transduction paths that control cell death could lead to novel therapies. Steady progress has been made in the translation of these early preclinical differentiation studies into clinical trials. At the beginning of the 1990s, one of the major successes was the finding that all trans-retinoic acid (ATRA) therapy led to major improvements in survival in patients with acute promyelocytic leukemia. Fittingly at the end of the decade, we saw that this success extends to solid tumors with the finding that retinoids significantly improved the long term survival of advance stage neuroblastoma patients. While past conferences centered on retinoids and leukemias as the major drugs and diseases of interest, this year's conference highlighted a number of drugs in preclinical development with novel mechanisms of action and a more concerted effort to evaluate the efficacy of these therapies on solid tumors.

## Clinical studies

Building on studies that showed more durable remissions could be achieved in APL using retinoids in combination with chemotherapy, R Ohno (Hamamatsu) presented the Japanese APL experience which confirmed previous complete remission rates (>90%) using a combination of all trans-retinoic acid (ATRA) and chemotherapy. Long-term disease-free survival was approximately 50%. These data mirror the long-term follow-up data from the North American Intergroup Study presented by M Tallman (Chicago, USA) indicating a 60% disease-free survival in patients treated with ATRA, either during induction, maintenance, or both. Although outstanding, they suggest ongoing room for improvement in outcome for patients with acute promyelocytic leukemia (APL) and emphasize the need for long-term follow-up of clinical trials in this disease. S Chen, Z Shen and Z Chen (Shanghai) presented exciting studies using arsenic trioxide for the treatment of APL. These studies confirmed the excellent response rate to arsenic trioxide in both relapsed and newly diagnosed patients. A significant proportion of patients (18/29 patients studied) treated with arsenic develop molecular remission (i.e. PCR-negative for PML-RARα transcript). Arsenic induction at 50% of the usual dose had a comparable response rate and may have less hepatotoxicity. Although there is general consensus that arsenic trioxide has extraordinary activity in this disease, there was no agreement among conference participants as to the best way to incorporate arsenic into APL therapy. In the current North America Intergroup study, patients are randomly assigned to two cycles of arsenic trioxide 'consolidation therapy' following ATRA/chemotherapy induction or an identical treatment regimen which does not include arsenic. R Warrell (New York) advocated the development of APL therapy that includes only ATRA and arsenic and avoids traditional cyctotoxic chemotherapy while L Degos (Paris) and Z Chen (Shanghai) currently reserve arsenic trioxide for relapsed patients.

P De Porre (Leuven), showed that Jannsen Pharmaceutical's R116010, a second generation retinoic acid metabolism blocking agent (RAMBA) was well tolerated in both normal volunteers and cancer patients. Preliminary evidence of clinical benefit was presented with several Phase I patients exhibiting stable disease. Endogenous serum retinoids appear doubled in volunteers taking R116010. P Adamson (Philadelphia) presented the results of the NCI Phase I study of the RAR and RXR agonist 9-cis-retinoic acid in a pediatric oncology population. As with ATRA, the chronic administration of 9-cis-retinoic acid appear to induce its metabolism through the p450 system. Dose-limiting toxicity was central nervous system, and maximum tolerated dose differed according to age group.

K Seiter (New York) presented data from a clinical trial in which patients with acute myeloid leukemia (AML) were randomized to receive 3 days of ATRA following induction chemotherapy or to receive chemotherapy in the absence of ATRA. Although no differences in outcome were seen between the two groups, bone marrow cells obtained following chemotherapy were more sensitive to the induction of apoptosis *ex vivo* in

response to ATRA than pre-treatment bone marrow cells, suggesting the potential utility of this strategy.

While the utility of differentiation therapy in the treatment of leukemias is now taken for granted, with few exceptions, its application to solid tumors has been slow in coming. Several reports indicated that steady inroads are being established in applying differentiation therapy to solid tumors. C Thiele (Bethesda) presented the exciting results from the CCG-3891 protocol for high-risk neuroblastoma. In a randomized trial of over 434 children and adolescents with newly diagnosed Evans Stage IV neuroblastoma, it was shown that patients receiving post-consolidation therapy with high dose 13-cis retinoic acid achieved a 46% 3-year event-free compared to 29% in controls.[1] R Pili (Baltimore) presented data in prostate cancer models suggesting that a combination of sodium phenylbutyrate plus 13-cis-retinoic acid led to increased apoptosis, inhibition of *in vivo* tumor growth, and inhibition of neo-vascularization compared to either agent used as monotherapy. B Spiegleman (Boston) discussed how activation of another member of nuclear hormone receptor family, PPAR$\gamma$, may have clinical use. PPAR$\gamma$ are fatty acid receptors important in adipocyte differentiation and the thiazolidinedione class of anti-diabetic drugs are agonists for PPAR$\gamma$. One of these, trogliatazone, was found to cause differentiation as assessed by an increase in lipid accumulation, a more normal histology and a decrease in a marker of proliferation in the liposarcomas of 9/11 patients. This class of receptors may be important in other cell types as well since E Mueller (Boston) demonstrated that PPAR$\gamma$ agonists inhibited the growth of both androgen-dependent and -independent prostate cancer cells causing a decrease in the production of prostate-specific antigen (PSA). Preliminary results from a clinical trial in patients with prostate cancer showed stabilization of PSA levels.

There was general agreement that well-designed clinical trials based on carefully developed pre-clinical models that included measuring important laboratory correlates should be pursued utilizing currently available drugs.

## Pre-clinical studies: Retinoids and other nuclear receptor

Pre-clinical approaches in APL have centered on novel retinoids, combination retinoid therapy and novel mechanisms of transcriptional regulation such as inhibitors of histone deacetylation. Y Jing (New York) observed a synergistic effect of the combination of all trans-retinoic acid (ATRA) and arsenic trioxide in NB4 cells both *in vitro* as well as in a SCID mouse model of APL. M Kizaki (Kei) found that the combination of GM-CSF and arsenic trioxide was a potent inducer of differentiation in both the ATRA-sensitive NB4 cells and the ATRA-resistant UF-1 cell line. Together these studies suggest that combinations of biologic response modifiers may have considerable therapeutic efficacy and might be particularly useful for ATRA-resistant APL. Foreshadowing experiments to come Z Chen (Shanghai) used microarrays to analyze the network of changes in gene expression stimulated by RA induced differentiation of NB-4 cells and compared these to the retinoid resistant but cAMP responsive NB4R1 cells. Among the incredible constellation of results presented was the interesting concept that retinoid induced differentiation may require activation of the protein kinase A path to induce differentiation.

The role of enhanced CD38 expression in triggering the retinoic acid syndrome in patients with acute promyelocytic leukemia was emphasized in an *in vitro* model presented by S Deaglio (Turin). CD38 interacts with CD31 on cultured endothelial cells triggering Ca fluxes and cytokine release by the activated CD38 positive cells. These events likely mimic the events associated with the activation of differentiating granulocytes that occurs in the retinoic acid syndrome.

Among the most exciting preclinical studies presented were the generation of the APL transgenic animals. P Pandolfi (New York) reported on the generation of transgenics containing each of the translocation chimeric proteins. 100% of the APL/RAR$\alpha$ transgenics acquire a myeloproliferative disease with 10% having tumors with a blocked differentiation phenotype. While 100% of the variant APL, PLZF/RAR$\alpha$ transgenics, develop a disordered leukemia at 6 months that looks more like CML than APL, those containing the reciprocal RAR$\alpha$/PLZF chimeric protein develop a myeloproliferative disease. Alone neither of the transgenics resembles APL, however 100% of the PLZF/RAR$\alpha$ $\times$ RAR$\alpha$/PLZF develop a leukemia with an APL phenotype. This demonstrates that both partners of the translocation are important in the development of the APL phenotype. The importance of such animals for pre-clinical drug development cannot be overstated especially with the ability to develop endpoints in assessing the efficacy of differentiation therapy. Preliminary results indicate that differentiation of APL cells can be seen in animals treated with Tricostatin A, an inhibitor of histone deacetylation and ATRA.

Clifford (Dallas) presented *in vitro* data investigating mechanism of retinamides. Retinamides affect appears dose related, causing rapid apoptosis at high (10 $\mu$M) doses, but slower induction of differentiation at lower doses (1 $\mu$M). Using wild-type F9 cells as well as F9 mutant lines that lack RAR, RXR, or both, they demonstrated that retinamide-induced differentiation is receptor-dependent, while retinamide-induced apoptosis is receptor-independent. Another novel retinoid in the pre-clinical pipeline included Targretin (LGD1069) which in the rat NMU breast cancer model could enhance the effects of tamoxifen at low doses and could cause regression in tamoxifen resistant tumors.

## HDAC inhibitors

In recent years our understanding of the basic principles of transcriptional regulation has evolved from a somewhat 2-dimensional view in which transcription factors recognize and bind to DNA sequences in the 5′ region of a gene to a more 3-dimensional view which ties transcription factors to higher

order chromatin complexes. C Glass (San Diego) presented the current working models in which RAR/RXR heterodimers interact in the absence of ligand with co-repressors (i.e. SMRT or sin3a) that have an intrinsic or associated histone deacetylation activity (HDAC) that may result in transcriptional repression. In the presence of ligand, RXR/RAR heterodimers from higher order complexes with co-activators (i.e. SRC, ACTR) and CBP/p300 that have a histone acetylation activity (HAT) that facilitates transcription. Whether this will hold true in all circumstances is unclear as S Mader (Montreal) showed that the effects of HDAC inhibitors on steroid signaling may be dependent on the relative intracellular levels of steroid receptors and other proteins that possess HAD and HAT activities. Complicating this picture even more was a presentation by L Delva (Paris) indicating that cellular retinoic acid binding proteins (CRABPII) associate in the nucleus with RAR/RXR heterodimers to form a complex that binds the RA-response element of target genes. These observations suggest the CRABPII may play an important and unexpected role in directly regulating transcriptional activation mediated through RAR.

M Yoshida (Tokyo) spoke about the identification of a number of agents such as trichostatin A (TSA), trapoxin TPX, FR901228 and MS-275 and a novel class of TSP/TPX hybrids that are potent inhibitors of histone deacetylation (HDAC). In some cases these agents cause cell cycle arrest, apoptosis or differentiation. V Richon (New York) spoke about the hybrid polar compounds and the mechanisms by which suberoylanilide hydroxamic acid (SAHA) acts as a potent HDAC inhibitor and induces the differentiation of MEL cells. L Butler (New York) demonstrated that SAHA was capable of significantly suppressing the growth of prostate cancer xenografts in nude mice with little side effects as measured by hematologic anomolies, body weight and histology. Using another hybrid polar compound, R Glick (New York) showed that m-caroxycinnamic bis-hydroxamide (CHBA) induced apoptosis in neuroblastoma cells that may be mediated by the FAS signaling system.

K Mills (Cardiff) identified the FUS (TLS) gene as being acutely downregulated during ATRA-induced granulocytic differentiation of HL-60 cells. He offered evidence that TLS expression was important in maintaining active proliferation in HL-60 cells since anti-sense TLS oligonucleotides inhibit proliferation and enhance the differentiation of these cells. This observation emphasizes the potential role of therapy targeted to specific (onco)genes for the treatment of malignancy. The appreciation that many hybrid transcription factors that result from chromosomal translocations can act as transcriptional repressors either directly or by interacting with transcriptional co-regulators suggests that these diseases may be amenable to therapies based on reversing transcriptional repression. A Melnick (New York) presented a detailed structure function analysis of the interaction between the POZ domain of the PLZF gene and the N-CoR transcriptional repressor. This and the finding that TSA plus ATRA can induce differentiation in the PLZF transgenic APL mice provide some of the most compelling preliminary evidence that the strategy of reversing transcriptional repression may be effective *in vivo*. Y Saunthatatajh (Bethesda) showed that phenylbutyrate and TSA were able to induce differentiation and apoptosis in t(8;21) AML carrying the AML/ETO fusion protein which is known to interact with the nuclear co-repressor N-CoR. Similarly, A Zelent (London) showed that TEL-AML1 can antagonize the activity of AML1 and since wild-type TEL can act as a transcriptional silencer it raises the possibility that the fusion protein may repress AML1 target genes. The finding that this TEL-AML1 activity is TSA sensitive suggests that transcriptionally targeted therapies may be important in treating t(12;21) cALL which accounts for almost 25% of all cALL.

## Manipulating apoptosis

Although a framework of apoptotic signaling paths has been established, the detailed integration of these paths with other metabolic paths as well as the regulation of initiation of apoptosis remains to be detailed. A Kimchi (Rehovot) used a functional anti-sense gene cloning approach to identify a number of death associated proteins (DAP kinase & DAP1-5). The anti-sense variants of these genes prevented interferon induced apoptosis in HeLa cells and encoded a variety of genes with diverse functions. DAP kinase is calmodulin-dependent serine theronine kinase that contains ankyrin repeats and a death domain containing protein that may act as a tumor or metastasis suppresser gene in some cancers. The novel DAPs encoded a variety of genes; DAP-1 encodes a small proline rich cytoplasmic protein; DAP-3 is a nucleotide binding protein; DAP-5 is a novel homolog of a translation initiation factor eIF4G. Now the challenge is how these new biochemical path integrate with other known inducers and suppressors of apoptosis. Using a similar approach A Kalvakolanu (Baltimore) isolated several genes associated with retinoid-interferon induced mortality (GRIM) of breast cancer cells. One of these GRIM-1 had three novel isoforms that were identified to be important in cell growth control and when over-expressed sensitized cells to RA+interferon induced cell death.

Using differentiation subtraction hybridization, P Fisher (New York) identified a number of genes induced when melanoma cells were induced to growth arrest and differentiate with interferon $\beta$. One such gene *mda-7* may have broader anti-cancer application as infection with an adenovirus expressing *mda-7* causes the growth arrest of a diverse number of tumor cell lines including glioblastoma, osteosarcoma and carcinomas with no discernable effect on normal epithelial or fibroblast cell lines. Preparations for a clinical trial using the Ad-mda-7 virus are in progress. Such functional gene mining experiments will be important not only in refining current cell death signal transduction paths because they have the potential to lead to the development of novel therapeutic approaches.

Arsenic has been found to be clinically important in treating relapse APL. Several groups detailed their analysis of the mechanisms of arsenic induced apoptosis. G Chen (Shanghai) presented evidence that apoptosis in arsenic treated APL cells is mediated via a disruption of the mitochondrial membrane while S Waxman (New York)

showed this may be dependent on glutathione and the activity of enzymes that regulate cellular $H_2O_2$ levels. R Pearse (New York) provided evidence that in multiple myeloma arsenic induced apoptosis may be mediated by activation of jun-kinase and the down-regulation of the anti-apoptotic gene NF-$\kappa$B.

D Kaplan (Montreal) showed intriguing results in which the proto-typical neural survival path NGF/TrkA can cause apoptosis in neuroblastomas and brain tumors. The high levels of TrkA obtained by infecting with a TrkA-adenovirus lead to auto-activation of the TrkA signal transduction path. Biochemical analysis indicates that activation of the MAPK path mediates these TrkA induced cell death signals. Since a number of companies and laboratories are screening biochemical and peptide libraries for agonists and antagonists of these signaling paths, it may be possible in the future to identify signaling paths that induce differentiation or cell death in tumor cells and then tailor the therapy to activate the appropriate paths to block tumor cell proliferation.

## Novel approaches

Sometimes a novel approach is just a different way of looking at the same old thing. While there were several reports that high doses of arsenate induce apoptosis in tumor cells *in vitro*, low concentrations were thought to have little effect. However, S Deaglio and G Baj (Turin) showed that when mammary tumor cells and myeloma cells were treated with low concentrations of arsenate there was a significant increase in CD54 (ICAM-1) expression and that these cells were now sensitive to LAK mediated cell killing. Furthermore they showed arsenate treated LAK cells were even more effective killers suggesting that arsenate may differentiate the tumor cells into a state more susceptible to immune surveillance and may also potentiate the immune systems anti-tumor activity. Another immunologically based differentiation therapy for human myeloid leukemia was presented by F Smadja-Joffe (Villejuif) indicating that anti-CD44 antibodies enhanced the *in vitro* differentiation of myeloid leukemia cells.

Several lines of evidence indicate that DNA methylation patterns are altered in tumor cells. Recent evidence indicates that over-expression of DNA methyltransferases (DNMT1) can cause cell transformation and inhibition of DNMT can block ras induced cell transformation. Surprisingly, DNMT induced transformation is not associated with changes in the pattern of methylated genes as tumor suppressor genes were inactivated prior to changes in methylation. M Syzf (Montreal) presented data indicating that aside from DNA methylation, the DNMT may associate with PCNA and be needed to fire origins of replication. Evolutionarily this would insure a coupling replication and DNA methylation. By inhibiting the DNMT, the firing of origins of replication was silenced and p21 and p53 were induced with p21 now able to bind PCNA and inhibit the cell cycle machinery. Such studies indicate that DNMT1 may be a novel target for therapeutic intervention in cancer therapy.

That differentiation based therapies are clinically viable is exemplified by the retinoid studies in APL and neuroblastoma. In the years to come the HDAC inhibitors and members of the nuclear receptor family hold promise for novel cancer treatments based on induction of differentiation. 'To be or not to be' that is still the question when a tumor cell encounters a novel chemical or biologic. Our challenge in more broadly applying these differentiation induction therapy strategies to the clinic is to better understand the mechanisms by which these agents induce differentiation or death.

## Reference

1. Matthay *et al.* (1999) New Eng. J. Med. 341: 1165 – 1173.