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*Meeting Report*

# New Insight on the Biology of Neuroectodermal Tumors

## Workshop Report from the University of Rome Tor Vergata and the IDI-IRCCS on the Genetics and Control of Growth, Differentiation, and Programmed Cell Death[1]

The second Workshop on the "Biochemistry of Neuroectodermal Tumors" was held in Rome on May 3–5 to review the most recent advances in the cell and molecular biology, and in the classical biochemistry, of tumors of neuroectodermal origin, and evaluate their clinical implications. The Rome meeting was convened by the University of Rome Tor Vergata and the IDI[2] to bring together the latest developments in the scientific understanding of these malignancies. Tumors of neuroectodermal origin, particularly small cell carcinoma of the bronchus (SCLC) and melanoma, are widespread in the adult population. Indeed, the incidence of melanoma is increasing rapidly, as was pointed out by R. Cavalieri (IDI, Rome, Italy) who detailed an alarming elevation in reported cases of melanoma in Italy, comparable to the 80% increase in reported cases in the United Kingdom over the last 5 years. Progress has been slow in delineating the key component in the tumoriogenic process in these tumors, perhaps because multiple genetic alterations contribute to the final tumorigenic phenotype. In the pediatric population NB is the second most common solid tumor in childhood and, as is the case with SCLC and melanoma, the treatment of the most frequent regional (stage III) and metastatic (stage IV) advanced stage NB is largely ineffective. In this brief report we can only select those aspects of the molecular genetics, the control of growth and differentiation, and the mechanisms of apoptosis that seemed of particular importance to the scientific committee.

### Genetics

The keynote lecture of the Rome meeting (The R. Sansone and S. Williamson Memorial Lecture) was given by Dr. C. Croce (Jefferson Institute, Philadelphia, PA), who, in summarizing the molecular genetics of hematological malignancies, showed how this approach can be applied to neoplasia in general. Amplification of an oncogene characteristic of NB, *NMYC*, has been known to be associated with advanced NB and poor prognosis for some years. Its discoverer, Dr. M. Schwab (Deutsches Krebsforschungs Zentrum, Heidelberg, Germany), showed how the transcriptional factor activity of the NMYC protein may be modified by its binding to a $M_r$ 20,000/22,000 doublet protein known as MAX. Dr. H. Shimatake (Toho University, Tokyo, Japan) related the heterogeneity of NMYC protein expression in neuroectodermal cell lines, tumors, and human embryos to the cell cycle phase and changes in microenvironment. *CMYB* is another oncogene expressed in NB, and Dr. G. Raschellò (Ente Nuove Tecnologie Energia ed Ambiente, Rome, Italy) showed that transfection with antisense CMYB oligonucleotides strongly inhibited NB cell proliferation *in vitro*.

Loss of heterozygosity on the short arm of chromosome 1 has also been known for some time to be frequent in NB, and Schwab refined the region of the putative NB tumor suppressor gene that is lost to the 1p36.1 region. After an 8-year search by numerous laboratories around the world, Dr. O. Delattre (Institut Curie, Paris, France) presented data on the identification and cloning of a region termed ESWR-1 on chromosome 22 that spans the t(11;22) (q24;q12) breakpoint detected in both Ewing's sarcoma and peripheral neuroepithelioma. The dissection of the t(11;22) (q24;q12) region, and the identification of two markers, SRPR and FL1, was described by M. Lipinski (Institut G. Roussy, Villejuif, France). Their finding that the translocation breakpoint for both Ewing's sarcoma and peripheral neuroepithelioma are clustered in the same region confirms the hypothesis proposed several years ago that these tumors were the same or ontogenically related. Closer analysis of the genes involved in the translocation breakpoint should lead to a better understanding of mechanisms of Ewing's sarcoma tumorigenesis which hopefully can result in innovative therapies for these tumors. Clearly, the mode of action of tumor suppressor genes is important, and Dr. S. Kovar (Children's Cancer Research Institute, St. Anna Kinderspital, Vienna, Austria) reported that mutations in the *p53* suppressor gene were frequent in Ewing's sarcoma and that clonal expansion of cells with *p53* mutations was important for metastasis. Alterations in *p53* were also noted by A. Iavarone and M. Israel (University of California, San Francisco, San Francisco, CA) in multifocal osteosarcoma and further functional studies identified a cellular protein that specifically bound wild-type but not mutant *p53*. Such studies should lead toward an understanding of the mechanism by which alterations in *p53* contribute to tumorigenesis.

### Control of Growth and Differentiation

Some tumors of neuroectodermal origin, particularly NB, can be induced to differentiate *in vitro* toward either a neuronal or epithelioid phenotype, although the latter pathway may also be associated with apoptosis. Dr. Levi-Montalcini (Consiglio Nazionale delle Ricerche, Rome, Italy) described the effects of NGF on a variety of neuroectodermal and skin cells, and the *trk* oncogene product, the high affinity receptor for NGF, was shown by P. Kogner (Karolinska, Stockholm, Sweden) to be preferentially expressed on NB of good prognosis. Neurotrophic factors, such as NGF, and their receptors, such as low affinity NGF receptor and *trk*, are differentially expressed in the developing rat inner ear (M. Saarma, University of Helsinki, Finland, and Tallin, Estonia), and in PC12 cells, in which they regulate the biosynthesis of various vesicle constituents during neuronal differentiation (C. Tschernitz, University of Innsbruck, Innsbruck, Austria). R. Slingerland (Academic Medical Center, Amsterdam, the Netherlands) described a uracil/cytosine nucleotide imbalance in PC12 cells by comparison with adrenal medulla, which appeared independent of growth rate. Drs. C. Thiele and C. Gaetano (National Cancer Institute, NIH, Bethesda, MD) discussed their work on the regulation of cell proliferation and differentiation in NB tumors and presented a model of NB cell differentiation in which induction of chromaffin differentiation occurred by increases in intracellular cyclic AMP, and RA-induced neuronal differentiation. RA inhibited cell cycle and growth-related genes, while dibutyryl cyclic AMP did not cause a similar shutdown. Thiele proposed that constitutive expression of the insulin-like growth factor II (a frequent occurrence in NB) blocked RA-induced differentiation.

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[2] The abbreviations used are: IDI, Istituto Dermopatico Immacolata; SCLC, small cell lung carcinoma; NB, neuroblastoma; IFN, interferon; RA, retinoic acid; NGF, nerve growth factor; IL, interleukin; VIP, vasointestinal peptide; TG, transglutaminase; TNF, tumor necrosis factor; PPI, phosphoinositides; VOCC, voltage-operated calcium channels.

A wide range of soluble factors with potential tumor growth-regulatory activity are elaborated by neuroectodermal tumors, and S. L. Lightman (Charing Cross Hospital, London, United Kingdom) and R. A. Knight (National Heart and Lung Institute, London, United Kingdom) showed that hypothalamic-releasing hormones and at least some ILs must now be added to the list. Moreover, since lymphocytes can also make neuropeptides and ILs, neuroectodermal tumors and their infiltrating lymphocytes share a common chemical language and each may influence the behavior of the other. Dr. G. Ludecke (University of Marburg, Marburg, Germany) showed, however, that ILs 1, 2, and 6 were without effect on NB growth *in vitro* although IFN-$\gamma$ did have some growth-inhibitory effects.

The expression of some peptides and neuropeptides in NB may be a useful method of imaging, refining clinical staging and therefore predicting prognosis. Kogner correlated elevated plasma neuropeptide Y with unfavorable, and elevated plasma VIP and somatostatin levels with favorable, clinical outcomes, data in accordance with the ability of VIP to differentiate NB cells *in vitro*. M. Maggi (University of Florence, Florence, Italy) identified and characterized two types of somatostatin receptors on NB cell lines. A specific high affinity receptor was only found on a subpopulation of NB cells with a relative immature phenotype, suggesting that tumor imaging with somatostatin may identify subpopulations of NB tumor cells with a relatively immature chromaffin phenotype.

## Neuroblastoma Metabolism

Muscarinic stimulation of NB cells elicits hydrolysis of both PPI and phosphatidylcholine, and A. Spinedi (University of Rome Tor Vergata, Rome, Italy) described differential removal of PPI- and phosphatidylcholine-derived diacylglycerol mainly by conversion to phosphatidic acid. S. Hrelia and B. Berra (University of Milan, Milan, Italy) proposed that PPI breakdown was a key signal in controlling proliferation, suggesting that transformation might be associated with aberrant regulation of protein kinase C by a diacylglycerol originating from a 4,5-$P_2$-phosphatidylinositol containing fatty acids different from arachidonic in position 2. On the other hand, NGF causes a rapid and transient activation of adenylate cyclase via GTPase in PC12 cells, possibly regulated by endogenous ADP ribosylation (S. Severin, Russian Endocrinological Research Centre, Moscow, Russia). The isoforms of GTP-binding proteins on NB cells at different stages of neuronal differentiation were described by M. Ponzoni (Gaslini, Genoa, Italy). Since the pattern of GTP-binding proteins was the same regardless of differentiation stage, these signal transducers are probably not involved in stage-related block of normal neuroectodermal cell development. M. Rutgers (Netherlands Cancer Institute, Amsterdam, the Netherlands) discussed the pharmacokinetics of *m*-iodobenzylguanidine in NB xenografts, with a view to optimizing therapeutic protocols.

Dr. F. Clementi (University of Milan, Milan, Italy) described $\omega$-type VOCC turnover in several NB lines, which was related to neurotransmitter secretion. VOCC activity during NB differentiation showed slower internalization and degradation, leading to an increased expression of membrane receptors, and reflecting more general phenotypic changes with respect to their secretory properties. Clementi's group also described neuronal nicotinic receptors on SCLC cell lines and L-type and $\omega$-type VOCCs on rat insulinoma cell lines.

G. Bruchelt (University of Tubingen, Tubingen, Germany) described the toxic effect of ascorbic acid on NB cells as mediated by $H_2O_2$ release through catecholamine metabolism, which, in the presence of free $Fe^{2+}$ released from ferritin as a consequence of the reducing power of ascorbic acid itself, allow the formation of highly cytotoxic $OH^{\cdot}$ radicals via the Fenton reaction. This mechanism of cell death may be open to pharmacological exploitation. Ten years' experience of using urinary catecholamines to detect early NB in infants through mass screening was summarized by T. Takeda (Sapporo National Hospital, Sapporo, Japan). Although 8 new NB cases of approximately 160,000 individuals screened have been found, doubts were expressed about the validity.

## Programmed Cell Death

Dr. L. Fesus (University of Debrecen, Hungary) reviewed the current status of programmed cell death (apoptosis) research highlighting the potential use of "tissue" TG (EC 2.3.2.13) as a biochemical marker of apoptosis. This physiological suicide program requires active metabolism and protein synthesis and is characterized by cell volume reduction inside an intact membrane, and degradation and condensation of chromatin in discrete masses. TG is specifically activated in apoptosis, catalyzing an acyl transfer reaction among polypeptide chains, and establishing $\epsilon$-($\gamma$-glutamyl)lysine and *N,N*-bis($\gamma$-glutamyl)polyamine isodipeptide linkages. This cross-linking of proteins forms the insoluble apoptotic body.

Drs. M. Piacentini and G. Melino (University of Rome Tor Vergata, Rome, Italy) reported RA-induced expression of TG (mRNA, protein, and activity); and their finding that an S (substrate-adherent) variant of NB has 28-fold higher TG expression than the neuroblastic (N) clones of NB makes it likely that S-type cells are on the pathway to apoptosis. The detection of TG mRNA but not protein in N-cell clones suggests that it is regulated at the translational level. Indeed, one point repeatedly and rightly emphasized at the meeting was the fallacy of inferring peptide concentration from mRNA expression.

## Melanoma

The variety of clinical types, and variety of etiological factors in malignant melanoma was emphasized by Dr. J. Di Giovanna (National Cancer Institute, NIH, Bethesda, MD). However, the roughly 1000-fold greater incidence of skin cancer in patients with xeroderma pigmentosum does highlight the etiological role of ultraviolet radiation causing DNA damage and the function of DNA repair as a protection mechanism. Some insight into the mechanism of melanoma metastasis was given by Dr. M. Capogrossi (National Institute of Aging, NIH, Bethesda, MD) who showed that interaction of melanoma cells, but not inert beads, with an endothelial monolayer led to a rapid rise in intracellular calcium in the endothelial cells. Moreover, P. G. Natali (Istituto Regina Elena, Rome, Italy) showed that increasing invasiveness of malignant melanocytes, in parallel to ICAM-1, integrin $\alpha 6/\beta 1$, laminin, collagen IV, and tenascin, is associated with progressive loss of the c-*kit* molecules, thought to be important in normal melanocyte migration and differentiation. Treatment of melanoma was discussed by Dr. E. Bonmassar (University of Rome Tor Vergata, Rome, Italy) who showed that xenogenization with triazene compounds of murine melanoma cells allowed them to induce enhanced antitumor immunity, and by Drs. C. Favalli and G. Garaci (University of Rome Tor Vergata, Rome, Italy), also working with a murine melanoma model, who showed how the combined treatment with prostaglandin $E_2$, 16,16-dimethylprostaglandin $E_2$ methyl ester, thymosin-$\alpha 1$, IFN, and IL-2 also increased the cytotoxic response, allowing perspectives toward human therapy.

## Conclusions

NB is a fascinating tumor to study, both because of the relative frequency of spontaneous regression *in vivo*, and also because of the wide availability of cell lines which can be induced to differentiate *in vitro*. Although studies on NB were less preponderant than in the First Biochemistry of Neuroectodermal Tumors Workshop, held 3 years ago, they still formed roughly 50% of the abstracts in this second

meeting. In the future, it is important to see how the elegant *in vitro* work in NB reported here can be confirmed on fresh tumor samples or on primary explants, and how many of the approaches pioneered in NB are applicable to other neuroectodermal tumors.

In NB, new markers, such as cell surface trk and secreted neuropeptide Y and VIP may be useful adjuncts to cytogenetics and *NMYC* amplification studies in clinical staging. In terms of therapy, worthwhile improvement may be achieved by using tumor necrosis factor and IFN to augment radioiodinated *m*-iodobenzylguanidine uptake, as reported by P. Cornaglia-Ferraris (Gaslini, Genoa, Italy).

With the recognition of the NMYC protein as a transcription factor, molecular genetic interest in NB is shifting toward analysis of tumor suppressor gene loss. Indeed, data from G. P. Tonini (Gaslini, Genoa, Italy) showed that loss of 1p is associated with more advanced clinical stage, and the result of cloning of this region in Schwab's laboratory is eagerly awaited. F. Lampert (Giessen, Germany) found 90% of NB tumors with intact and normal complement of chromosome 1 have a good prognosis, while in NB tumors with alterations or deletions of chromosome 1, only 30% have a good prognosis. Cell cycle analysis of NB tumors also indicates that patients with aneuploid tumors and *NMYC* amplification should receive more aggressive chemotherapy (J. Benard, Institut G. Roussy, Villejuif, France). Similarly, molecular analysis of the chromosome 11 and 22 breakpoint regions will contribute to the production of a complete molecular interpretation of NB and Ewing's sarcoma.

While the molecular understanding of other neuroectodermal tumors lags behind, its development is being stimulated by the experimental possibilities exemplified by NB. We look forward to the next Biochemistry of Neuroectodermal Tumor Workshop, to be held in Rome in 1995, in anticipation not only that the analysis of NB will be still further advanced, but that studies on other neuroectodermal tumors will be catching up fast.

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