

**Session Reports from the
XVIIth International Pigment Cell Conference
and Satellite Meetings**

Index

Satellite Meeting No. 1 - Vitiligo: A Manifestation of Apoptosis?
PS1 - Biochemistry of Melanogenesis, Melanosomes, and Melanocytes
CS2 - Chemistry and Biophysics of Melanin & Melanogenesis
PS2 - Basic Biology of Melanoma
CS3 - Melanoma: Ultraviolet Light, Diagnosis and Treatment.
PS4 - Inter/Intracellular Signaling Pathways in Melanocytes
CS5 - Melanocyte Photobiology/Chemistry and UV protection
CS6 - Ocular/Extracutaneous Melanin and Melanogenesis
Hot Topics Symposium
CS8 - Control of Hyper/Hypopigmentation
Satellite Meeting 4 - Sentinel Node mapping and dissection in the Staging and Treatment of Melanoma

Satellite Meeting No. 1

Vitiligo: A Manifestation of Apoptosis?

Report by James J. Nordlund

Symposium Chairmen: James J. Nordlund (University of Cincinnati) and Seung-Kyung Hann (Korean Institute for Study of Vitiligo and Yonsei University)

Goal of Symposium: This symposium on vitiligo emphasized one mechanism, i.e., apoptosis. The goal was to stimulate new ideas and approaches to the problem of vitiligo and its treatment.

Major Problems for Investigators: (James J. Nordlund, M.D., University of Cincinnati, USA)
The most important goal for investigators is to find a safe, effective medication that will stop the spread of vitiligo when it first begins or that retains pigmentation after successful treatments. The second most important goal is to find better ways to transplant autologous melanocytes in those whose depigmentation affects glabrous (hairless) skin.

Mechanisms of Cell Death:

Ultrastructure of apoptosis: (Raymond Boissy, Ph.D., Caroline LePoole, Ph.D. and Ying Boissy, University of Cincinnati) Apoptosis is a programmed induced destruction of cells by which unneeded or senescent cells are eliminated from the body without induction of an immune process. Necrosis is another form of cell death by which the cells are disrupted and release cellular antigens that might induce an immune response.

Apoptosis has morphological features including 1) alterations in membrane fluidity; 2) blebbing of the plasma membrane; 3) cell fragmentation in which apoptotic bodies are

released into the environment; 4) vacuolization of the cytoplasm; 5) cellular shrinkage; 6) condensation of the chromatin along the nuclear membrane; 7) activation of calcium or potassium sensitive endonucleases that fragment DNA.

Apoptosis has been demonstrated as a mechanism of melanocyte death in a model system in which human melanocytes in culture are exposed to chemical toxins such as 4-tertiary butyl phenol (4-TBP). Melanocytes obtained from patients with vitiligo that are grown in culture also exhibit features of apoptosis especially if the cells are not maintained in very special conditions.

Apoptosis has not been clearly observed in the skin of patients with advancing vitiligo. However some melanocytes in the spreading lesion exhibit features that suggest cellular shrinkage and condensation of heterochromatin, features consistent with apoptosis.

Molecular Mechanisms of Apoptosis: (Yoshihide Tsujimoto, Ph.D., Osaka University, Japan) Apoptosis is a method of destruction of cells that must be selectively eliminated as part of tissue homeostasis, for morphogenesis or removal of other harmful cells such as cancers. The process is driven by a family of cysteine proteases called caspases. Caspases cleave a set of special proteins that initiate apoptosis. The Bcl-2, Bcl-x and Bcl-w are three of the better characterized anti-apoptotic proteins that inhibit apoptosis and protect a cell from destruction. Pro-apoptotic molecules include molecules such as Bid, Bik and Bim. The anti and pro apoptotic proteins form heterodimers inactivating each other.

Mitochondria play an essential role in apoptosis. The electrical potential across a cell destined for destruction by apoptosis is lost and cytochrome c and other factors are released. Bcl-2 prevents cytochrome c release, whereas Bax, Bak and Bid accelerate these processes.

Cytochrome c activates caspase 9 and ultimately caspase 3 releasing cytochrome c through a permeability transition pore. The pro and anti apoptotic factors enhance or close pore permeability respectively. A newly discovered factor, Acinus, induces chromatin condensation after cleavage by caspase-3.

Apoptosis and necrosis share some common steps. The concentration of ATP within a cell is one factor that determines whether a cell undergoes necrosis or apoptosis.

Immune Mechanisms of Apoptosis: (Tatsuya Horikawa, M.D. and David Norris, M.D., University of Colorado) Cells exposed to toxins can die by either apoptosis or necrosis. Severe damage induces necrosis, intermediate damage initiates apoptosis. Many of the immune activated processes induce apoptosis such as TNF-a or TNF-b. Cytotoxic lymphocytes induce apoptosis through three distinct mechanisms. 1) coordinate release of perforin and granzymes adjacent to the target cell membrane; 2) cytokine release (i.e., IFN-g, TNF-a, IL-1); 3) triggering of the Fas receptor.

Cells maintain complex anti-apoptotic defenses such as Bcl-2. Interruption of these defenses by immune mechanisms facilitate apoptosis in susceptible cellular targets. When activated cytotoxic lymphocytes encounter cells expressing high levels of ICAM-1, they bind to and engage Fas on the target surface. Those cells susceptible to apoptosis undergo programmed cell death. Others are destroyed by necrosis.

Apoptosis is a key mechanism controlling the balance among the highly changing interactive cell populations in the immune response. Activated lymphocytes expressing Fas ligand on their cell surface induce apoptosis of lymphocyte and macrophage targets bearing Fas. Apoptosis is a complex process involving multiple mechanisms of induction. But Fas-dependent lysis of cytotoxic T lymphocytes is the most efficient and highly developed form of immune cytotoxicity.

Mechanisms of repigmentation:

Melanocyte reservoir: (James J. Nordlund, University of Cincinnati) Data show almost conclusively that depigmentation of vitiligo is caused by loss of melanocytes. Histological data confirm absence of cells by routine, histochemical, and ultrastructural techniques. Attempts to culture cells from depigmented skin have been unsuccessful.

Glabrous skin (hairless skin) or depigmented skin with white hair do not respond to medical therapies for repigmentation. These areas do repigment by surgical techniques. These data indicate that the hair follicle is the normal reservoir for repigmentation and that surgical transplants are a method to replace the follicular reservoir.

Mechanisms of melanocyte proliferation and survival: (Zalfa Abdel Malek, Ph.D., University of Cincinnati) Melanocytes can be induced to proliferate in response to stimuli such as ultraviolet light.

Many growth factors have been identified that stimulate growth of melanocytes. Some are synthesized in the epidermis, some in the dermis. Factors such as b-FGF, endothelin-1, α -MSH and ACTH are synthesized by keratinocytes and melanocytes.

Proliferation of melanocytes requires the activation of multiple signaling pathways. Together cAMP and activation of protein kinase C induce melanocyte mitosis. Endothelin-1 has a complex action including activation of protein kinase C, calcium mobilization and inositol triphosphate formation. These factors subsequently activate map kinase erk 1/erk 2 and the transcription factor CREB (calcium/camp response element binding protein) which are necessary for proliferation.

Endothelin 1 also enhances melanocyte motility and reduces adhesion to fibronectin. Mutations in receptors for endothelins such as endothelin-3 are responsible for a syndrome characterized by absence of both melanocytes and enteric neurons. Mutations in the c-kit gene are responsible for piebaldism.

Challenges for the future: (Seung-Kyung Hann, Vitiligo Research Center and Yonsei University). The causes and cures of vitiligo have remained elusive. It should be a disease of great importance to pigment biologists because the disorder provides clues to the normal function of the pigment cell with the other epidermal cells, the keratinocyte and Langerhans cells to form the KLM complex.

In this symposium we have suggested that apoptosis might be a mechanism by which the melanocytes are destroyed and we have reviewed the factors that can cause the cells to return to the depigmented skin. It is hoped that these presentations will be the stimulus for more discussion and conversations among pigment biologists to find new ways to solve the problem of vitiligo and its cure. One idea worth presenting is that there are weak and strong melanocytes in the skin of a given person. By identifying why some cells are stronger and resist vitiligo, we will find ways to prevent or halt the spread of vitiligo. When the human genome is mapped, it is hoped that the genes involved in vitiligo will be identified and that rapid progress on the cause and cure of this disease will be made.

-

PS1 - Biochemistry of Melanogenesis, Melanosomes, and Melanocytes

Report by John Pawelek

Dr. K. Jimbow presented the opening lecture "Signals and Molecules Involved in Melanosome Biogenesis and Melanocyte Differentiation". Melanosomes share many biological properties with lysosomes. Early, stage I melanosomes appear to be linked to endosomal vacuoles.

Melanosomal proteins are glycosylated and are assembled through interaction with calnexin in the ER, eventually exiting through the trans Golgi network to the final melanosome compartments. Adapter protein-3 is important in intracellular transport of tyrosinase gene family proteins. Also, small GTP-binding proteins, e.g. Rab 5 and 7, as well as phosphatidylinositol 3-kinase regulate traffic. Thus, the cascade of melanosome biogenesis is regulated by several factors: 1) glycosylation of melanosomal proteins and correct folding and accumulation within the ER and Golgi, and 2) specific signals and molecules involved in intracellular transport and vesicular traffic of these glycoproteins from the Golgi to melanosomes.

Using HPLC and X-ray microanalyses, Smit et al. studied the relative proportions of pheo- and eumelanins in melanocytes from dark- and light-skinned individuals. Light-skinned individuals have a higher level of pheomelanins and lower level of eumelanins than those with darker skin. These differences can be accentuated if the cells are cultured with excess L-tyrosine. Cultured nevus cells from patients with dysplastic nevus syndrome tend to produce predominantly pheomelanins, and again this phenomenon is enhanced if the nevus cells are cultured in high levels of tyrosine.

Solano et al. presented "New Insights on the Murine Silver Locus: On the Expression of the Transcript in Wild-Type and Silver Mutation and Function of the Protein". Wild type mouse cells express a gp87 silver locus protein that, though smaller, is similar to human gp100, whereas silver mutant cells express a truncated version of gp87, due to a premature stop codon at nucleotide 1808. Studies on the oxidation of DHICA by extracts of mutant and wild-type cells suggested that DHICA oxidation is catalyzed by TRP-1, and apparently not gp87.

Kobayashi and Hearing continued their studies on the stabilization of tyrosinase through binding interactions with TRP-1, and here demonstrated direct interaction of the two proteins *in vivo*. Employing a chemical cross-linker to stabilize the association of tyrosinase with other cellular proteins, they showed by immunoblotting that TRP-1 was indeed associated with tyrosinase in amounts much greater than could be accounted for by nonspecific cross-linking. Together, the data indicated that TRP-1 interacts directly with tyrosinase, stabilizing it *in vivo*.

Toyofuku et al. studied aberrant folding and transport of mutant tyrosinases in type I oculocutaneous albinism (OCA1). Computer models suggest that some amino acid substitutions in OCA1 may alter folding of tyrosinase, but it is not yet clear why this eliminates tyrosinase activity. Accordingly, wild-type and OCA1 mutant tyrosinases were transfected into COS7 cells. Some mutant tyrosinases were retained in the ER and not transported to lysosomes, the final destination of tyrosinase in cells other than melanocytes. Tyrosinases with temperature-sensitive mutations were transported appropriately to lysosomes, but not expressed at physiological temperatures. It was concluded that some aa substitutions altering the 3-D structure of tyrosinase result in temperature-sensitivity of the enzyme, while others result in trapping of mutant enzymes in the ER, preventing transport to melanosomes.

Peters et al. reported the presence of POMC-related peptides in melanosomes of cultured human melanocytes. Probing with specific antibodies they reported cross-reactivity with alpha MSH, proconvertases 1 and 2 (PC 1 and 2) and the PC2 regulatory protein, 7B2. Thus they identified elements of the POMC system in melanosomes, providing supportive evidence for their model of complex formation between MSH and co-factor 6BH4, which activates tyrosinase by relieving 6BH4 inhibition of the enzyme. Such a model suggests that MSH action does not necessarily involve MSH receptors.

CS2 - Chemistry and Biophysics of Melanin & Melanogenesis

Report by Patrick Riley

This session began with a Keynote Lecture given by the Chairman of the Conference, Professor Shosuke Ito. His talk, entitled "Chemical Analysis of Melanins and its Application to the study of Melanogenesis Regulation" began with a detailed review of a range of studies using the assay introduced in 1983 as a means of estimating the relative amounts of phaeomelanin and eumelanin. This assay is based on the conversion of DHICA moieties in eumelanin to the derivative pyrrole tricarboxylic acid (PTCA) by permanganate oxidation in approximately 2.8% yield, and benzothiazine moieties (in phaeomelanin) to 4-amino-3-hydroxyphenylalanine (AHP) by hydriodic acid hydrolysis in 20% yield under defined conditions of treatment of melanin samples. Professor Ito suggested that the proportional generation of phaeomelanin, assessed by these criteria, is inversely related to the level of tyrosinase activity. Application of this model to murine coat colour mutants was considered to be consistent with this general proposal. Professor Ito further suggested that the inner region of polymeric melanins consists of phaeomelanin with an outer layer composed predominantly of eumelanin. In response to questions Professor Ito suggested that at very low levels of tyrosinase activity any dopaquinone generated is rapidly converted to cysteinyl-dopa and eliminated from cells resulting in the absence of detectable melanin. With regard to the structural model of melanin proposed it was at the molecular level and would not be easily discernible by techniques such as electron microscopy.

Professor Patrick Riley then presented a talk entitled "The Source & Significance of Dopa in Phase I Melanogenesis" which outlined the overwhelming accumulation of evidence favouring the indirect generation of dopa by reduction of dopachrome in the initial steps of *in vitro* melanogenesis. He argued that this mechanism, coupled with the reduction of active site copper atoms, was able to account for the observed 'lag phase' kinetics of tyrosinase. In response to a question from Professor Frank Meyskens he agreed that UV-induced changes in tyrosinase synthesis may be significant but expressed doubts concerning the activation of tyrosinase by superoxide anion generated by UV exposure. In his view the intracellular proton concentration was more likely to be a significant regulator of tyrosinase recruitment.

The next talk was given by Professor Tadeusz Sarna in which he outlined the functions of the retinal pigment epithelium (RPE) in providing metabolic support for photoreceptor cells. He drew attention to the decrease in RPE melanization as a function of age. Experiments on melanosomes obtained from differentially melanised bovine eye RPE demonstrate that photo-oxidation is more pronounced in the absence of melanin. Thus, decreasing retinal melanization may have a bearing on pathological conditions such as age-related macular degeneration.

Dr. Jun Matsunaga then presented evidence from clonogenic assays that a short (30-minute) exposure of B16 or Neuro2A cultures to selected melanogenic intermediates exhibited differential cytotoxicity. Of the agents tested the most toxic were dopachrome and dopaminochrome. This finding was interpreted to imply an important cytoprotective role for dopachrome tautomerase activity as demonstrated for migration inhibitory factor (MIF).

Dr. Malgorzata Rozanowska presented data from pulse-radiolysis and laser flash-photolysis experiments showing that carotenoid cation radicals are quenched by synthetic melanin. In other experiments it was shown that light- or chemically-induced lipid peroxidation was abrogated by the addition of a series of carotenoids and that their efficiency was increased in the presence of melanin.

Finally, Dr. Jeffrey Tosk gave an ebullient account of studies of Rayleigh scattering by suspensions of dopa melanin. Using a polarised light scattering technique the effects of microenvironmental alterations, such as pH and redox state, on the aggregation of the pigment could be demonstrated. These effects were considered to be relevant to the association between pigmentation and neuronal damage observed in Parkinson's disease. In response to a question from Professor Raimondo Crippa he agreed that the aggregation state may regulate the extent of the reactive surface of melanin.

PS2 - Basic Biology of Melanoma

Report by Frank Meyskens

The sessions on "Basic Biology of Melanoma" was represented by six platform and eleven poster presentations and covered a wide range of topics including keratinocyte / melanocyte/melanoma cross-talk and its importance in invasion, immunogenic properties of melanosomal proteins, angiogenesis, control of reactive oxygen species, and molecular genetic alterations.

Herlyn (USA) gave a superb overview of the topic of keratinocyte/melanocyte and biochemical interactions and regulation and its importance in maintaining tissue integrity. Substantial evidence was offered that the replacement of E-cadherin by N-cadherin during melanocyte transformation is a critical event that permits invasion to occur. Using a reconstructed model system MacNiel

(England) offers evidence that erosion of the basement membrane was necessary for invasion by melanoma cells and that this process was reduced by 17 β -estradiol and estrogen. This observation is, of course, of great interest since longstanding clinical data clearly shows that young women with melanoma have a much better prognosis, even after adjusted for Breslow level. Related to this general topic were two papers on angiogenesis: Both calponin (Koganchiua, Japan) and α 5 β 3 integrin (Kageshita, Japan) respectively were found to be altered expression in the blood vessels and melanoma cells. As is being documented by many investigators the role of angiogenesis is crucial in the pathophysiology of carcinogenesis and many new therapies are being developed based on such fundamental observations.

Papers were presented by four different groups (Kawasaki, Japan); Bartido, USA; Cochran, and Pawelick, all USA, New Haven) demonstrating the complexity of response of immune responses in melanocyte biology. Melanosomal proteins, dendrite cells, and T-lymphocytes all play a role and in a manner too complex to review here. Perhaps, the most intriguing of the observations is the necessary evidence being amassed by Pawelek & his group that metastatic melanoma cells may be a hybrid of host (macrophage) and melanoma cells. If this hypothesis can be proven, the theoretical and practical consequences would be enormous for tumor biology.

A group of papers also dealt with various aspects of biochemical controls including the inter-relationships between tyrosinase, trp-1, and d-glutamyltranspeptidase (Chasuble, India), various oncogenes products

(Ueda, Japan) and the role of a -PKC in phospholipase alterations. Two complex papers (Takara, Japan; Andrs Germany) on the genetics of melanoma were presented as well.

Two posters were presented on the general topic of reactive oxygen species. Haycock (England) nicely demonstrated that a -MSH blocked the acute TNF or peroxide stress response in melanoma cells which has implications for the study of early inflammatory changes in melanocyte carcinogenesis. Our own group (Spillane, U.S.A) has presented the unique observation that apoptosis of melanoma cells is promoted by heavy ion chelators, inhibited by classical antioxidants, and that ferrous (anti-apoptotic) and ferric (pro-apoptotic) ions change the intracellular redox state in an opposing manner and affect apoptosis accordingly.

CS3 - Melanoma: Ultraviolet Light, Diagnosis and Treatment.

Report by Alistair J Cochran

CHAIRS: Alistair J Cochran, Bertil Kagedal, Juichiro Nakayama

Papers presented:

Understanding Regulation of NFκB as Basis for Melanocyte Transformation and Melanoma Cell Therapeutic Resistance, Frank L. Meyskens Jr., Susan McNulty, Niloufar Tohidian, and Julie Buckmeier, Chao

Effective Early Detection of Malignant Melanoma on the Sole, Toshiaki Saida,

Does PCR Detect Melanomas in Sentinel Nodes (SNs) that are Not Detected by Immunohistochemistry? J. Guo, J. Messina, D.-R. Wen, R.R. Huang, D. Reintgen, and A.J. Cochran,

Primary Cutaneous and Vulvar Melanomas are Dissimilar in Clinical and Histopathological Features, but Similar in TP53 Mutations, Boel Ragnarsson-Olding, Anton Platz, Sofia Karsberg, Lena Kanter-Lewensohn, and Ulrick Ringborg,

This session brought together a series of interesting if somewhat diverse papers.

Dr Meyskens reported that melanoma cell lines have elevated levels of reactive oxygen species relative to normal melanocytes. He and his colleagues studied nuclear factor kappa B (NFκB), a transcriptional regulatory complex that responds to reactive oxygen species and ultraviolet B, as a potential factor in melanoma progression and chemoresistance. UVB increases NFκB binding by normal melanocytes, but not by melanoma cell lines. Oxidative stress induces increased NFκB binding in both melanocytes and melanoma cell lines. The NFκB response of melanoma cells to UVB is apparently independent of oxidative stress and is mediated at the transcriptional level.

Dr Saida reported melanocytic nevi, visible as brownish black macules, on the soles of 8% of Japanese. These must be separated from early melanomas which may appear quite similar. With the exception of congenital nevi, most nevi on the sole are less than 7mm in diameter, leading to a recommendation that lesions larger than 7mm be excised. In contrast most melanomas are larger than 9mm in diameter. Epiluminescence microscopy (ELM) may provide further assistance in making this separation. Patterns seen on ELM that may be used to separate melanomas and nevi were reported. An algorithm for the management of these lesions has been developed.

The paper by Guo et al. described a study of the tumor status of sentinel lymph nodes (SN), comparing standard histology (SH), immunoperoxidase (IPX), RT-PCR (PCR), using a primer for m-tyrosinase and RT in situ PCR (ISPCR) (same primer). A series of 15 SN was studied. Five were positive for tumor by SH, IPX and PCR, 5 were negative for tumor by these three techniques and 5 were negative by SH and IPX, but positive by PCR. Additional sections were cut from the blocks of these 15 nodes and studied by ISPCR, using a primer for m-tyrosinase. In the 5 nodes that were negative by SH, IPX and PCR, ISPCR detected no tumor, but found neural tissue (Schwann cells containing m-tyrosinase) in 4 of 5 and a capsular nevus in one. In the 5 nodes positive by SH, IPX and PCR, ISPCR confirmed the presence of melanoma, but additionally found neural tissues in three. In the 5 nodes that were negative by SH and IPX, but positive by PCR, ISPCR demonstrated no melanoma, but all five contained neural tissue and two had a capsular nevus. It was concluded that sources of PCR signal for m-tyrosinase other than melanoma cells are present in lymph nodes. This must be considered in interpreting results from PCR studies of complex tissues such as lymph nodes.

Dr Ragnarsson-Olding reported a comparison of malignant melanomas (MM) arising on sun-exposed and non sun-exposed (vulvar) body sites and, within the vulva, on hair-bearing and glabrous skin. From a study of 219 patients the density of MM on the vulva is 2.5 times that on other areas of the body. On the vulva MM are often amelanotic. Mucosal lentiginous MM was most common, followed by nodular and superficial spreading melanoma, the reverse of the frequency on non-vulvar sites. In a study of TP53 mutations in 35 MM, 27% of those from sun-exposed and 34% from non-exposed sites displayed the C-T mutations at dipyrimidine sites that are considered fingerprints of UV radiation-induced DNA damage. It was concluded that the similarity in frequency of the TP53 mutations in MM from exposed and non-exposed sites points to a complex genesis for these alterations. The clinical and histologic differences between melanomas of the two sites are striking but inexplicable at this time.

Papers by Cascinelli and by Lee et al. were withdrawn

PS4 - Inter/Intracellular Signaling Pathways in Melanocytes

Report by Glynis Scott

The session on inter/intracellular signaling pathways in melanocytes was opened by Dr. Nishikawa who spoke about the microenvironment control of melanocyte migration and localization to the hair follicle. Their studies on the role of stem cell factor (SCF), c-kit and endothelin-1 show that SCF and ET-1 are critical growth factors for the localization of melanocytes to the skin. Their studies show that E-cadherin levels increase in melanocytes prior to entry of the melanocytes to the epidermis, and that this increase in expression is highly synchronized. They also show that overexpression of SCF through the use of transgenic mice results in population of melanocytes in the interfollicular areas. Dr. Nishikawa and co-workers show that the soluble and the membrane bound forms of SCF have differing functions in hematopoietic and future work in their laboratory will focus on defining the effects of the soluble Vs membrane bound SCF on melanocyte migration and localization to the skin.

Dr. Thody and co-workers presented data on the regulation of melanocyte function by α -MSH. Dr. Thody reviewed findings confirming the importance of the MC-1 receptor in mediating the diverse effects of α -MSH on skin pigmentation. He then reviewed data showing that other POMC peptides, such as ACTH, are available ligands for the MC-1 receptor in the skin and may indeed have differing effects on melanocyte function compared with α -MSH. He shows that ACTH peptide 1-17 coupling to cAMP is greater than that seen with α -MSH and that certain ACTH peptides activate IP3 production. Another point of potential regulation is the levels and activity of proconvertase enzymes, which convert PMC to ACTH. Future studies will involve defining the dose response curve for POMC peptide effects on melanocyte function including melanogenesis, dendricity and adhesion, and the signaling intermediates involved. Dr. Medrano presented data on the effect of pigmentation associated genes on cell cycle and terminal differentiation in human melanocytes. Dr. Medranos' laboratory has made the observation that activation of cAMP results in senescence of human melanocytes and that senescence is accompanied by other markers of differentiation such as increased pigmentation. Their data indicates that changes in the levels of E2F/p130 repressor complexes is associated with terminal differentiation and increased pigmentation, suggesting a possible mechanism for increased melanoma susceptibility in light skinned individuals who cannot tan. Dr. Medrano also shows that cyclin E is markedly decreased in senescent cells, whereas it is increased in primary melanoma. Future work will be focused on defining the potential regulatory sites at which pigmentation associated genes affect cell cycle.

Dr. Tuma presented data on the role of the microtubule motor protein kinesin II and the actin binding protein myosin V on melanophore movement in *Xenopus*. By using dominant negative mutants of these two proteins fused to green fluorescent protein they show that cells

expressing both mutants fail to exhibit any melanophor movement whatsoever. In cells expressing only the dominant negative kinesin mutant, melanophores failed to demonstrate the usual quick saltations towards the periphery, but did show slow, random movements. Cells expressing the dominant negative myosin V mutant showed some movement of melanophores to the periphery, but melanophores fail to remain at the periphery. These results demonstrate the importance of these two proteins in melanophore movement and future studies will be directed to understanding the regulation of these motor proteins in melanophore movement.

Dr. Tada presented data on the signal transduction intermediates involved in ET-1, α -MSH and bFGF signaling in human melanocytes. A main point of their presentation is that CREB activation may occur through two distinct signaling pathways: one that is mitogen activated and ERK2 dependent and one that is stress induced and p-38 dependent. They show that ET-1 and bFGF, but not α -MSH, induce CREB phosphorylation, and that ERK2 activation is necessary for ET-1 induced CREB phosphorylation. Ultraviolet light also resulted in CREB activation, but through phosphorylation of p38 rather than ERK2. These results highlight the importance of CREB phosphorylation as a signaling intermediate in mitogen induced proliferation/differentiation and in response to stressors such as UV light.

Mr. Wagner presented the final talk in the session from Dr. MacNeils laboratory. In the studies presented the investigators demonstrated the effect of α -MSH on Ca^{+2} mobilization. Using the human melanoma cell line HBL as a model system, the investigators show that when cells were cultured on fibronectin, and when the α -MSH induced cAMP response was inhibited by an adenosine agonist, α -MSH induced increased intracellular Ca^{+2} levels in the majority of cells studied. This dual response of the MC-1 receptor to α -MSH is similar to that observed with the MC-3 receptor found in the brain. Future studies will be directed at determining the biological significance of these observations.

CS5 - Melanocyte Photobiology/Chemistry and UV protection

Report by Zalfa Abdel-Malek

Chairpersons: Zalfa Abdel-Malek, Masamitsu Ichihashi, Mauro Picardo

The melanocyte plays a central role in the cutaneous photobiological response to sun exposure. This response is determined to a large extent by constitutive pigmentation and subsequently by the tanning ability that confers photoprotection against further sun-induced DNA damage.

Gilchrist and Eller described the melanogenic response of S91 melanoma cells to the introduction of small DNA nucleotides into these cells. These DNA fragments enhanced DNA repair and resulted in activation of the p53 pathway, which includes induction of p21, PCNA, XPA, XPC, GADD45, and tyrosinase.

Hill and Hill emphasized the differences in the responses of melanoma cells synthesizing different types of melanin (eu- versus pheomelanin) to different UV spectra. Their presentation also emphasized the importance of comparing survival curves, as well as mutagenicity following exposure to different UV spectra. The conclusion was that melanin may be a sensitizer or a photoprotector depending on the UV wavelength, form of melanin, and the assays used to determine the function of melanin.

The correlation between skin phototype and antioxidant levels are evaluated following sun exposure by Picardo et al. Although SOD/catalase ratio correlated with the MED, it was found

that a specific allelic form of catalase is expressed at a high frequency in low skin phototypes.

Moro et al. reported that the differential sensitivity of melanocytes, keratinocytes, and fibroblasts to ultraviolet radiation (keratinocytes=fibroblasts>melanocytes) might explain the higher incidents of basal and squamous cell carcinoma than melanoma, and the photoaging response to sun exposure. Keratinocytes are also more sensitive than melanocytes to the cytotoxic effect of sodium arsenite, which results in basal and squamous cell carcinoma, hyperpigmentation, but not melanoma.

Smit et al. showed that in cultured human melanocytes, formation of cyclobutane dimers and 6,4-photoproducts in response to UVB light correlated directly with melanin content. However, no significant differences were found in the rates of DNA repair or in survival among melanocytes with different melanin contents.

CS6 - Ocular/Extracutaneous Melanin and Melanogenesis

Report by Dan-Ning Hu

A Symposium on Ocular/Extracutaneous Melanin and Melanogenesis was chaired by Dan-Ning Hu and Shosuke Ito. Dan-Ning Hu (USA) delivered the keynote lecture. The methods for cultivation and studying uveal melanocytes have been established in the past 10 years. Human uveal melanocytes grow well and produce melanin *in vitro*. The growth and melanogenesis of cultured uveal melanocytes are regulated by various biologic substances. The stimulators consist of some growth factors (etc. bFGF, HGF), Endothelin, adrenergic agonists and various prostaglandins. The inhibitors consist of TGF β , cholinergic agonists and IL-6. The uveal melanocytes are similar to epidermal melanocytes except in their response to α -MSH and ACTH. Both hormones stimulate the growth and melanogenesis of epidermal but not uveal melanocytes in cAMP-elevating agents deleted medium.

Giuseppe Prota (Italy) and Hu's work on types of ocular melanin was presented, they found that the iris pigment epithelium contains mainly eumelanin. Iridal melanocytes contain both eumelanin and pheomelanin, the ratio being variable depending on iris color. The pheomelanin/eumelanin ratio is higher in growing cultured uveal melanocytes than those in senescent cells. Kazumasa Wakamatsu (Japan) used an improved alkaline peroxide oxidation method to study neuromelanin. They found that neuromelanin consists mostly of dopamine with 10-20% incorporation of cysteine and that its structure is rather complex as compared to synthetic melanin.

Raymond Boissy (USA) reported that α -MSH does not stimulate the growth of uveal melanocytes *in vitro* and they could not detect receptors of α -MSH in uveal melanocytes by northern blot analysis and immunocytochemical studies. Khaled Abul-Hassen (UK) reported the transfer of gene to retinal pigment epithelium by a liposome-based method and to uveal melanocytes by adenoviral vector. After gene transfer, tyrosinase activity of melanocytes responds to various factors, but pigment epithelium lacks a factor for post transcriptional and/or translational modification of tyrosinase. Joan Roberts (USA) reported that cultured uveal melanocytes eliminate exogenous nitric oxide, an important regulator in ocular, neural and vascular tissues. Clearance of nitric oxide is directly related to melanin content. In the discussion, S. MacNeil (UK) mentioned that although α -MSH does not simulate growth and melanogenesis of cultured uveal melanocytes, intracellular calcium is elevated by α -MSH when the cell is cultured on fibronectin and adenosine agonist is added into the medium.

Hot Topics Symposium

Report by Vince Hearing

This Symposium was an innovation of the XVIIth IPCC and attempted to give a special forum to the most interesting and exciting abstracts submitted. As you know, all abstracts were judged blindly by the cochairs of each Symposium to select the best 3 to be presented orally in that Symposium. The highest scoring abstract from each topic was then considered for the 'Hot Topics Symposium' and the top 5 were selected for this special forum (in other words, it was quite an honor to present here not to mention that fact speakers got an extra 5 minutes).

Robert Hoffman (a PASPCR member) presented the first paper and reported his group's attempts towards regulating pigmentation using a *tyrosinase* gene encapsulated within liposomes for delivery to follicular melanocytes. The *tyrosinase* gene used was from *Streptomyces* and was linked with an internal ribosome entry site to allow expression (the bacterial enzyme was used rather than the mammalian enzyme because of its significantly smaller size). Albino mouse skins in organ culture were used as targets and some hair repigmentation was observed following this gene therapy approach. Since hair and skin melanocytes are relatively accessible to such targeting, the potential use of such gene therapy is a tantalizing goal for correcting pigmentary defects. A similar approach using antisense strategy would potentially down-regulate pigmentation, and their future research is aimed at increasing the efficiency and application of this approach.

Bernhard Wehrle-Haller next reported his group's work on stem cell factor (SCF), an important paracrine signaling factor for melanoblast survival. Various segments of the cytoplasmic tail of SCF were deleted in order to characterize the role(s) of various motifs on intracellular processing and secretion by keratinocytes. As an approach to identify where the mutant SCFs went, he incorporated green fluorescent protein (GFP) into the extracellular part and used fluorescent microscopy to track their localization. Wild-type GFP-SCF was transported to the cell surface where c-kit (the receptor) positive melanocytes would bind to it. Deletion of various segments of the cytoplasmic tail identified a short sequence required for this specific processing and distribution. That sequence is completely conserved in avian and mammalian SCF and Dr. Wehrle-Haller postulates that this motif is required for SCF function and correct melanoblast localization during development. In co-culture experiments, melanocytes and melanoblasts actively associated with wild-type SCF expressing fibroblasts, but not with mutant SCF expressing fibroblasts, leading to the hypothesis that SCF is not only important for localization of melanoblasts towards SCF-positive cells, but also that it might play other roles in cross-talk between these types of cells, perhaps even facilitating melanosome transfer.

Masashi Kato next described their further studies with the *ret* transgenic system in which *ret*-overexpressing mice have high rates of spontaneous melanoma generation. He now reported that the increases in *ret* expression were accompanied by increases in MAP kinases and c-Jun, as well as in matrix metalloproteinases. Their group proposes that increased expression of *ret* disrupts normal intracellular signaling which may play a role in malignant transformation of melanocytes. He used this melanoma system as an appropriate model to examine the efficacy of agents targeted against melanoma, and he reports that an herbal medicine (active component = ???) delayed the onset of tumors, and significantly decreased metastasis of melanomas to various organs.

Miri Seiberg (another PASPCR member) reported interesting studies that are attempting to sort out factors responsible for melanosome transfer to keratinocytes. She reports that they have recently found that the protease activated receptor-2 (PAR2) is important to this exchange, and that agents which inhibit or stimulate the function of PAR2 have dramatic effects on melanosome transfer in vitro, and also in vivo in a Sinclair swine model. PAR2 is expressed on the surface of keratinocytes (not melanocytes) and seems to play an important role in regulating melanocyte-keratinocyte interactions. Her results further suggest that

increased transfer has a feedback mechanism that stimulates the melanocytes to produce more melanin, and vice-versa. Physical contact between melanocytes and keratinocytes is essential for these effects and co-culture of those cells separated by a permeable membrane prevented those effects. This study is an important first step in understanding mechanisms involved in the physical transfer of pigment granules from melanocytes to keratinocytes, a process that heretofore has essentially been poorly understood.

Shigeru Sato presented the final paper in this session, which dealt with the characterization of the mutation in *Mitf* found in the *Black-Eyed White* mouse. As we know, *Mitf* not only plays an important role in the regulation of melanoblast development, but also plays an important role in regulating differentiation of functional melanocytes. Null mutants of *Mitf* have a complete loss of pigment cells in all normally pigmented tissues, but the *Black-Eyed White* mutation is an interesting one wherein mutant mice have a normally pigmented retinal pigment epithelium, but lack melanocytes in the skin and inner ear. This mutation was shown to result from an insertion into intron 3 of *Mitf*, which affected the distribution of *Mitf* isoforms. *Mitf* is now known to be transcribed in 3 active forms, *Mitf*-A (predominantly expressed in the RPE), *Mitf*-H (predominantly expressed in the heart) and *Mitf*-M (predominantly expressed in non-RPE melanocytes). The *Black-Eyed White* mutation affects the processing of these isoform mRNAs, leading to only slight decreases in *Mitf*-A and *Mitf*-H, but to complete down-regulation of *Mitf*-M, which explains its dramatic effects on melanocytes in the skin and inner ear, and lack of effect on RPE. This study demonstrates the critical nature of the *Mitf*-M isoform for normal melanocyte development.

In sum, this Hot Topics Symposium was a novel venue to highlight the most topical and important abstracts submitted to the IPCC. It will be interesting to see if this becomes a traditional feature of future IPCC.

CS8 - Control of Hyper/Hypopigmentation

Report by Ray Boissy

The development of facultative cutaneous pigmentation is under the regulation of extramelanocytic factors generally produced by the keratinocytes of the epidermis, that can in turn be modulated by the environment, predominantly by ultraviolet irradiation. In order to begin to assess the facultative development of skin hyper/hypopigmentation analyses using co-cultures of melanocytes and keratinocytes is beginning to be exploited. Presentations in this seminar focused on such model systems. "Keratinocyte-melanocyte co-cultures and pigmented reconstructed epidermis as models to study skin pigmentation and its modulation" presented by R. Schmidt began the session. He reviewed techniques developed at L'Oreal to screen for inhibitors of melanization. He specifically discussed the following. [a] Cultured melanocytes are routinely developed using a medium reported by Dr. Olsson of Sweden designed for the culturing and expansion of vitiligo derived melanocytes. [b] Total cellular melanin was recovered from sonicated cells using a DEAE cellulose filter. [c] Keratinocyte-melanocyte co-cultures were established using a 10:1 ratio in keratinocyte specific medium resulting in successful melanosome transfer. [d] A technique using C¹⁴-thiouracil to assess rate of melanin synthesis was developed. [e] A solar simulator was used to irradiate melanocyte cultures and co-cultures in which the threshold for increased melanogenesis was lower in the latter condition demonstrating that keratinocytes are a major mediator of UV induced cutaneous hyperpigmentation. [f] A reconstructed skin equivalent model was presented which was utilized to investigate the effects of sunscreens and UVR, Kojic acid, IBMX, and melanocytes from various ethnic donors on pigmentation of the model system. M. Kim presented "Modulation of melanin neosynthesis by plant extracts in human keratinocyte-melanocyte co-culture system" in which a Transwellâ system was used to test for diffusible molecules mediating the keratinocyte regulation of melanocytes. They demonstrated that the

hypopigmentary effect of two specific plant extracts occurred only in the co-culture system and not in pure melanocyte cultures. The keratinocyte affect appeared to be modulated by IL-1a, IL-5 and IL-6. A. Hachiya presented "A paracrine role of stem cell factor/c-kit linkage in UVB-induced pigmentation" which investigated the expression of SCF and c-kit in co-cultures. They demonstrated that transcript and protein expression of SCF (the membrane bound form only) and c-kit were upregulated by UVB in a dose dependent manner. This upregulation was confirmed immunocytochemically *in vivo*. In addition, using a guinea pig skin system, UVB-induced hyperpigmentation could be inhibited by subepidermal injection of anti-c-kit antibody.

Melanocyte stimulating hormone and the agouti protein are also extramelanocytic regulators of cutaneous pigmentation via the receptor, MC1R. Z. Abdel-Malek presented "The melanocortin-1 receptor is a key regulator of human cutaneous pigmentation" in which she reviewed data demonstrating that MSH and ASP could increase and decrease respectively the activity of cultured human melanocytes in response to ultraviolet irradiation. Significant data was presented demonstrating that cultured human melanocytes homozygous for an Arg160Trp mutation in the MC1R failed to respond to the effects of both MSH and ASP. ASP is a ten fold larger molecule than MSH. V. Virador presented "Bioactive domains and expression patterns of the agouti signal protein" in which the ASP was molecularly dissected to identify its bioactive domain. Synthetic 15mer segments of the ASP were assessed for their ability to reduce melanization of cultured melanocytes. A region containing KKVARRPR, just outside of the basic domain of ASP, was most effective. A model was presented suggesting how ASP binds to the MC1R to alter the pocket used for MSH binding. In addition, a synthetic peptide (a PEP16) was developed which demonstrated reactivity in hair follicles consistent with ASP being secreted by keratinocytes.

Successful cutaneous pigmentation is the result of the transfer of melanosomes from the dendrite tip of the melanocytes to the neighboring keratinocytes. This process has remained molecularly elusive. G. Scott presented "Dendrite formation and melanosome transfer - beyond the electron microscope" which clearly demonstrated in transfection studies that a member of the Rho family of molecules associated with actin assembly (i.e., Rac 1) is involved as a downstream regulator of dendrite extension. UVR and MSH can also influence rac-1 function. Rac 1 function appears to be regulated by TIAM-1. Analysis of sucrose density purification of melanosomes demonstrated expression for specific SNARE attachment protein receptors for vesicle fusion (i.e., SNAP 23, SNAP 25, syntaxin 4, and VAMP-2) along with a SNARE associated docking protein (Rab 3a). These molecules putative mediate the fusion of the melanosome with the plasma membrane of the melanocyte and its transfer through the plasma membrane of the recipient keratinocyte. Regulation of these molecules may ultimately underlies the development of hyper/hypopigmentation of the skin and hair.

Satellite Meeting 4

Sentinel Node mapping and dissection in the Staging and Treatment of Melanoma.

Report by Alistair J Cochran

Chairs: Alistair J Cochran, M.D., FRCP, FRCPath, and Toshiaki Saida, M.D., Ph.D.

Meeting Program:

Lymphatic Mapping and Sentinel Node Surgery, Alistair J. Cochran, M.D., FRCP, FRCPath, Department of Pathology and Laboratory Medicine, 13-145 CHS, UCLA School of Medicine, Los Angeles, California, 90095.

Sentinel Node Dissection for Melanoma, Richard Essner, M.D., FACS, Assistant Director of Surgical Oncology, John Wayne Cancer Institute, Santa Monica, California.

The role of Lymphoscintigraphy and Other Radionuclide Techniques in the Localization of Sentinel Lymph Nodes, E.C. Glass, R. Essner, A.J. Cochran, Haigh, D. Morton, John Wayne Cancer Institute, Santa Monica, California.

Occult melanoma micrometastases in the regional lymph nodes: Their detection, distribution and clinical implication, Minoru Takata and Naohito Hatta, Department of Dermatology, Kanazawa University School of Medicine, Kanazawa, Japan.

Clinical Experience with the Sentinel Node Technique in Japanese Patients with Melanoma, Naoya Yamazaki National Cancer Center Hospital, 5-1-1, Tsukiji, Chuo-Ku, Tokyo 104-0045, Japan.

Clinical Experience with the Sentinel Node Technique in Non-melanoma Skin Cancers, Yoshio Kiyohara, Masahito Taguchi, Tadashi Suzuki, Tetsuya Tsuchida, Department of Dermatology, Saitama Medical School, Saitama, Japan

Dr Cochran presented the background and significance of techniques that were the subject of the meeting. The management of high-risk (deep, thick) primary melanoma, clinically localized to the primary site, has been unsatisfactory. Treatment options were elective nodal dissection, an operation with substantial morbidity, that is probably unnecessary for 80% of patients or a policy of "wait and see" that delays definitive treatment for the 20% of patients who eventually recurred. Lymphatic mapping and the selective removal and close evaluation of the sentinel node (SN) provides a more rational approach. The symposium brought together experts who were active in originating and developing the techniques and clinicians involved in their application. Areas covered included localization of the SN, evaluation of the SN for tumor by histology, immunohistology and molecular techniques and the treatment and prospects of patients with tumor-positive SN. Current clinical experience in the US and Japan was presented.

Dr Essner described the surgical aspects of the approach noting that success requires close cooperation by skilled surgical, nuclear medicine and pathology personnel. There are three separate components: mapping of the direct lymphatic route from the primary site to the regional lymph node basin, identification of the first lymph node in that basin that receives lymph via the direct lymphatic (the sentinel node) and selective excision of the SN, leaving the non-sentinel nodes in place. Complete lymph node dissection is reserved for individuals with tumor in the SN, those most likely to benefit from node dissection. Identification of the SN originally depended on visualization of the afferent lymphatics and target node based on their blue color after injection of a blue dye (isosulfan blue). The technique is more accurate and easier to master when the blue dye is combined with preoperative identification of the lymphatics and SN by early read (dynamic) lymphoscintigraphy and intraoperative detection of the SN by a hand-held gamma probe. It was stressed, however, that the technique requires an initial learning period during which the performance of surgeon, pathologist and nuclear medicine personnel will improve quite rapidly. Since its development by Morton et al (1992) the effectiveness of the approach has been widely validated by other workers. The technique has also been applied to many other tumors, such as breast cancer, vulvar cancer and colon cancer. Lymphatic mapping and sentinel lymph node evaluation is at least as efficient as elective node dissection as a staging technique and may be superior as it permits the pathologist to focus on a limited number of nodes and apply special approaches, such as immunohistology and molecular pathology to this limited target. Two major studies are in progress. The Multicenter Selective Lymphadenectomy Trial randomizes patients to receive either wide local excision or wide local excision and lymphatic mapping and selective lymphadenectomy. If the sentinel node contains tumor the patient receives a complete lymphadenectomy. The Sunbelt Melanoma Trial is examining the role of SN evaluation in

determining patients likely to benefit from treatment with interferon-alpha. From the results of these trials the therapeutic role of selective lymph node dissection will become clear.

Dr Glass provided an overview of the role of the nuclear medicine physician. Preoperative lymphoscintigraphy (LS) is essential to avoid the errors implicit in clinical assessment of likely lymphatic drainage patterns, to chart the route of the afferent lymphatic and to identify the SN within the relevant nodal basin. The site of the SN can then be marked on the skin to facilitate placement of the surgical incision. Preoperative LS requires the intradermal injection of radiopharmaceuticals such as Tc99m antimony trisulfide colloid, Tc99m albumin nanocolloid or Tc99m sulfur colloid. Optimal agents feature rapid migration times, good retention in the SN, minimal spillover into non-sentinel nodes and low radiation dose to the patient. After intradermal injection imaging with a scintillation camera should begin immediately to capture images of the afferent lymphatics and SN. Body outlines and the position of the SN are to be clearly marked to assist the surgeon in localizing his approach. The optimal arrangement is to undertake surgery within two hours of LS, obviating the need for a second perioperative injection of isotope. Unusual drainage paths are not uncommon. Special attention is to be paid to cervical, facial, epitrochlear, popliteal, umbilical and scapular nodes.

Problems encountered by new users are most often due to relatively simple factors. These include the use of excessive tracer (>18MBq), too long a period between tracer injection and imaging, poor positioning of the patient, failure to mark the outline of the body and failure to accurately mark the position of the SN on the skin. With attention to detail and moderate experience satisfactory results may be expected.

Dr Cochran outlined the role of the pathologist who, once the surgeon and nuclear medicine physician have identified and removed the SN, has the critical responsibility of determining whether the SN contains tumor. A single SN will be provided from 67% of patients, two SN from 25% and three or more from the remaining 12%. Because of the relative difficulty of interpreting frozen section material and the likelihood of losing diagnostic material because of distortion during freezing and thawing and tissue removal to obtain "full face" sections the use of frozen sections is not recommended. Sentinel nodes are carefully bisected through their longest circumference and ten serial full-face sections removed from each cut face. Sections 1, 3, 5 and 10 are stained with hematoxylin and eosin, section 2 with S100 and section 4 with HMB-45. Tumor cells are to be separated from capsular and trabecular nevus cells, paracortical dendritic cells, macrophages and intranodal nerves. It is essential to use immunohistochemistry to avoid missing up to 24% of positive SN. In numerous studies between 18-20% of SN have been found to contain tumor. The risk to personnel from technetium 99 at the doses used is considered slight, but standard radiation precautions should be utilized. Studies with molecular biology approaches are of great interest, but have not yet become standard. In providing material for experimental studies pathologists should divide the tissues in such a manner that the diagnostic process is not compromised.

Drs Takata and Hatta reported a study of 436 nodes from 32 patients. HMB-45 detected tumor in the nodes of 15/24 patients who were negative by HE. In 23 nodes nested PCR for tyrosinase was more sensitive than a combination of single round PCR for the melanoma associated markers tyrosinase, MART-1, Pmel-17, TRP-1 and TRP-2. Tyrosinase mRNA was detected in 6 nodes that were negative by HE and HMB-45.

Using HMB-45 and nested RT-PCR for m-tyrosinase, maps of distribution of microscopic and "submicroscopic" metastases were generated for 13 inguinal basins. The authors reported considerable variation in the distribution patterns of occult metastases in the node basins studied. The authors conclude that the main role of molecular biology is to identify the subgroup of patients whose nodes are negative by histology and molecular biology and who require no additional treatment.

Dr Yamazaki reported on clinical experience at the National Cancer Center Hospital, Tokyo. In a study of 14 patients with acral lentiginous melanoma, using the dye patent blue V alone,

the SN was identified in 13/14 cases. Sensitivity and accuracy were similar to those reported previously and the authors found the approach "feasible and successful."

Dr Kiyohara and his colleagues reported their experience with the techniques in the management of 24 patients with non-melanoma skin cancer (squamous cancer, porocarcinoma, invasive Bowen's disease and invasive Paget's disease) at Saitama Medical School. In 11 cases the SN contained tumor and a complete lymph node dissection was undertaken. In no case was a non-sentinel node found positive in the presence of a negative sentinel node. A single sentinel node was found in 76% of patients and two in 24%. The authors conclude that the approach is "reasonable and reliable."

References: Morton, DL, Wen, D-R, Wong, JH, Economou, JS, Cagle, LA, Storm, F.K., Foshag, LJ, Cochran, AJ: Technical details of intraoperative lymphatic mapping for early stage melanoma. Arch Surg. 127;392-399, 1992.