

CONFERENCE
ON
Multiple Hereditary Exostoses
Insights Into Pathogenesis

November 3-5, 2005

Shriners Hospital of Houston
6977 Main Street
Houston, Texas

and the

Houston Marriott Medical Center
6580 Fannin Street
Houston, Texas

Organizers: Dan Wells, Ph.D., Jacqueline Hecht, Ph.D., Sarah Ziegler

Sponsored By:

The Shriners Hospital
The National Institutes of Health
American Association of Enchondroma Diseases
March of Dimes Birth Defects Foundation
The Orthopaedic Research Society
The MHE Coalition
Gene Dx, DNA Diagnostic Services
The Mizutani Foundation for Glycoscience

**HME: Insights into Pathogenesis.
Schedule of Events**

Thursday November 3, 2005

8:30-9:00 Arrive at Shriners (Coffee and Pastries)
9:00-9:10 **Dan Wells, Ph.D.** Welcome to conference
9:10-9:30 **Jacqueline Hecht, Ph.D.** Introduction to genetics and natural history MHE

ORTHOPAEDICS AND CLINICAL GENETICS (Christine Alvarez)

9:30-10:10 **Christine Alvarez, M.D.** Introduction of orthopaedics and defining severity of Hereditary Multiple Exostosis
10:10-10:40 **Luca Sangiorgi, M.D., Ph.D.**
Mutational analysis and clinical expression of disease in patients with HME.
10:40-11:00 **BREAK**
11:00-11:30 **Wim Wuyts, Ph.D.**
Mutation detection strategies for molecular screening in patients with HME.
11:30-12:00 **George Thompson, M.D.**
Clinical treatments and surgical procedures.
12:00-1:15 **LUNCH**

BONE DEVELOPMENT (Dan Wells)

1:15-1:45 **John R. Hassell, Ph.D.**
FGF binding to percelan isolated from the growth plate.
1:45-2:15 **David Ornitz, M.D., Ph.D.**
FGF that regulate growth plate development.
2:15-2:45 **Maurizio Pacifici, M.D., Ph.D.**
Syndecans: Cell surface modulators of growth plate chondrocytes
2:45-3:15 **T. Michael Underhill, Ph.D.**
Delineation of regulatory networks underlying BMP action in chondrogenesis
3:15-3:30 **BREAK**
3:30-4:00 **Henry Kronenberg, M.D., Ph.D.**
How PTHrP regulates chondrocytes in the growth plate.
4:00-4:30 **Andrea Vortkamp, Ph.D.**
Ihh signaling in the growth plate.
4:30-5:00 **Matthew Hilton, Ph.D.**
Multiple Aspects of Ihh Signaling in the Endochondral Skeleton.
5:00 **Transport back to hotel**
6:30- **Leave for Dinner at Las Alamedas**

Friday November 4, 2005

8:30-9:00 Arrive at Shriners (Coffee and Pastries)

EXOSTOSIS/DEVELOPMENT (Jacqui Hecht)

- 9:00-9:30** **Jacqueline Hecht, Ph.D.**
Analysis of human exostoses and derived cells.
- 9:30-10:00** **Jeffrey Esko, Ph.D.**
Signaling defects in chondrocytes give rise to exostoses
- 10:00-10:30** **Dominique Stickens, Ph.D.**
Mice deficient in EXT2 lack heparan sulfate and develop exostoses.
- 10:30-10:45** **BREAK**
- 10:45-11:15** **Pancras Hogendoorn, M.D., Ph.D.**
Profiling of osteochondromas and peripheral chondrosarcomas.

EXT FUNCTION AND NON-MAMMALIAN MODELS (Scott Selleck)

- 11:15-11:45** **Marion Kusche-Gullberg, Ph.D.**
EXT1 and EXT2 proteins and heparan sulfate biosynthesis
- 11:45-12:15** **Scott Selleck, M.D., Ph.D.**
Heparan sulfate biosynthesis and sulfation.
- 12:15-1:30** **LUNCH**
- 2:00-2:30** **Catherine Merry, Ph.D.**
ES Cells and the role of Heparan Sulphate in Development and Disease.
- 2:30-3:00** **Rahul Warrior, Ph.D.**
Developmental regulation of HSPG synthesis during *Drosophila* development
- 3:00-3:30** **Malorzata Wiweger, Ph.D.**
Zebrafish as a model for studies on Hereditary Multiple Exostosis
- 3:30-3:30** **BREAK**

NON-BONE PHENOTYPES (Yu Yamaguchi)

- 3:30-4:00** **Yu Yamaguchi, M.D., Ph.D.**
Phenotypes of conditional EXT1 knockout mice: Non-skeletal symptoms.
- 4:00-4:30** **Jeremy Turnbull, Ph.D.**
Structure and biosynthesis of mouse brain heparan sulfate.
- 4:30-5:00** **Harish Hosalkar, M.D.**
Keloid Formation Following Surgical Treatment of MHE.
- 5:00** **Transport back to hotel**
- 7:00-** **Banquet at Hotel**

Saturday November 5, 2005

8:30-9:00 Continental Breakfast at Hotel

HME-RELATED DISEASES (Benjamin Alman)

- 9:00-9:30 Benjamin Alman, M.D.**
p53 and Rb in Cartilage Tumors in Mice.
- 9:30-10:00 Roland M. Leach, Ph.D.**
Tibia Dyschondroplasia; Ihh signaling.
- 10:00-10:30 Pancras Hogendoorn, M.D. Ph.D.**
Olliers and Mafucci.
- 10:30-11:00 James Martin, Ph.D.**
Malignant transformation in human chondrosarcoma cells.
- 11:00-11:30 Meeting Summary and Review (Wells and Hecht)**
- 11:30-1:00 LUNCH**

ABC's of MHE WORKSHOP (Sarah Ziegler)

- 1:00-1:30 Jeffrey Esko, Ph.D. and Scott Selleck, M.D., Ph.D.**
Overview of the scientific sessions of the conference.
- 1:30-2:00 Wim Wuyts, Ph.D.**
Genetics of HME.
- 2:00-2:30 Jacqueline Hecht, Ph.D., and Sandra Darilek, M.S.**
Presenting study results, Hereditary Multiple Exostoses and Pain.
- 2:30-2:45 BREAK**
- 2:45-3:15 Ashish Sinha, M.D., Ph.D.**
Chronic pain and the need for treatment
- 3:15-3:45 Harish Hosalkar, M.D., John P. Dormans, M.D.**
ABC's of MHE.
- 3:45-4:15 John E. Herzenberg, M.D., FRCSC**
Use of Fixators.
- 4:15-5:00 Questions and Discussion**
- 5:00- Buffet Reception**

Session 1

**Thursday November 3, 2005
9:30am - 12:00pm**

**ORTHOPAEDICS AND
CLINICAL GENETICS**

Christine Alvarez, Chair

Defining the Severity of Hereditary Multiple Exostosis

Christine Alvarez

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Introduction: Hereditary Multiple Exostoses (HME) is a genetic disorder with a wide phenotypic expression involving limb alignment, limb lengths and height, lesion factors, and potential for sarcomatous transformation. The clinical impact of HME is likely related to number of lesions a patient has. It remains unclear what factors are predictive of the number of lesions an individual becomes riddled with. However, these factors are likely related to the patient's genotype. HME results from mutations in the exostoses genes: EXT1 and EXT2. The function of these genes involves controlling physal chondrocyte proliferation and differentiation. Mutation of either gene results in loss of control of the physal maturation gradient which eventually leads to excess chondrocyte proliferation and presumptive osteochondroma formation. EXT 1 has a more dominant role in this mechanism and therefore an EXT 1 mutation would likely have greater impact and result in a greater loss of chondrocyte regulation in the zone of proliferation and thus, more potential for exostosis formation.

Previous work examining the potential for a genotype phenotype relationship showed that EXT1 patients showed a worse phenotype. This study however had a small sample size and though trends in many of the 58 phenotypic parameters tested were identified statistical significance was not uniformly met. Other studies also have found a similar relationship of EXT 1 being worse phenotypically but phenotypic parameters were incomplete, in some studies the sample was still small, and in some cases genotypic was based on linkage analysis only and not definite mutations. Thus, a more descriptive and extensive clinically significant evaluation of these patients is required as well as a study involving a greater number of patients to provide sufficient power to address issues of statistical and clinical significance.

Purpose:

The purpose of this study was to build on the original studies on genotype phenotype correlation in HME. This project was designed to establish an extensive phenotype profile, define genotype and have large sample size. This study will then therefore define the genotype phenotype relationship in HME.

Materials and Methods:

Genotyping was performed at the Molecular Diagnostic Laboratory at BC Children's Hospital. EXT1 and EXT2 mutations were analyzed by direct sequencing of entire gene coding regions, including flanking intronic sequences. Phenotyping was performed at the HME Clinic and involved collection of radiographic and clinical parameters divided across three categories of lesion quality, limb segment lengths, and limb alignment.

Results:

Sixty-eight affected individuals with HME participated in the study. Genotype data was collected for all participants. Phenotype data including lesion number and quality (17 parameters), limb segment lengths (6 parameters x 2 side), longitudinal height, and limb alignment (28 parameters) were collected. Baseline demographics were established considering number of patients affected with EXT 1 vs. EXT 2 mutations, gender, and age (classified into four age categories: 0-5, 6-10, 11-15 and +16 years). The primary premise, that more lesion cause worse severity, was established, and then applied to the principal hypothesis that EXT 1 mutations produce a worse phenotype.

Mutational analysis of EXT1 and EXT2 genes and a new system for “grading” clinical expression of disease in patients with Hereditary Multiple Exostoses.

¹Luca Sangiorgi, ²Nicola Fabbri, ²Laura Campanacci, ¹Elena Pedrini, ¹Veronica Maini, ¹Silvia Capponcelli, ¹Marina Mordenti and ²Mario Mercuri.

¹Genetics Unit, ²V Surgery Division, Rizzoli Orthopedic Institute, Bologna, Italy.

Introduction: Several mutations dispersed throughout the EXT1 and EXT2 genes have been identified in HME patients. Most of these are responsible of the truncation of EXT1 or EXT2 proteins which are likely to become inactive and degrade rapidly, resulting in a nearly complete loss-of-function. A prospective longitudinal study was undertaken to correlate genotype and phenotype of the disease in families and sporadic cases, with the purpose of defining the disease spectrum of expression.

Method: A multidisciplinary clinic, involving geneticists and orthopaedic surgeons, is weekly carried out at the Rizzoli Institute. In order to evaluate the spectrum and distribution of mutations leading to HME in the Rizzoli cohort of patients, a new multi-step DHPLC-based mutation screening method was optimised. For informative families, we adopted a pre-screening linkage analysis to selectively focus the DHPLC testing on either EXT1 or EXT2. Patient clinical assessment is carried out using a new classification system substantially based on deformity and functional limitation and the genotype-phenotype study is performed using this system.

Results: In a small pilot we enrolled 36 unrelated probands, representing 20 families and 14 sporadic cases for the presence of mutations in either EXT1 or EXT2 genes. Thirty-one out of 36 probands (86%) had mutations either in EXT1 (24/31; 77%) or EXT2 (7/31; 23%), mainly distributed in the amino-terminal region of the proteins and the vast majority of them are responsible of a premature protein truncation. Most mutations were distributed towards the 5' end of EXT1 and EXT2 genes. In accordance with the most recent data, 23 of 31 mutations (74%) were novel. No novel missense or splice site mutations were detected in 200 control chromosomes. All splice site mutations were present in the highly conserved AG and GT positions of the splice acceptor and donor junctions, and only one of these (c.IVS6+1G>T in the EXT1 gene) had been previously reported in the HGMD database. The 3 missense mutations clustered within exon 2 of the EXT1 gene and caused amino acid substitutions in residues 339 and 340. Two of these (c.1018C>T (p.R340C), c.1019G>A (p.R340H)) had been already described whereas the third one (c.1016G>T (p.G339V)) was a novel mutation responsible for substitution of a glycine codon with a valine within an important key element for EXT1 function. The overall mutation frequency in 36 probands was 86% (67% in EXT1 and 19% in EXT2). Mutations were found in 10 of 14 sporadic cases (71%) and in 20 of 21 familial cases (95%). No disease-causing mutation has been detected in 5 of 36 patients. A new clinical classification based on clinical evaluation and orthopaedic problems has been defined. A preliminary study performed on 153 pts have shown that most (80%) are not substantially limited by the disease. Using the DHPLC based screening technique and the clinical classification system, we are currently running a genotype/phenotype study on 170 patients. Preliminary results indicate that mutations on specific exons of the EXT1 gene correlate with more relevant clinical limitations.

Conclusions: Our results identified several novel and many private mutations in a large cohort of patients, confirming the strong allelic heterogeneity of EXT1 and EXT2 genes. Our optimised DHPLC-based approach represents a reliable, efficient and highly sensitive diagnostic strategy for rapid detection of germline mutations in HME patients. The genotype-phenotype study is giving indication regarding association of alterations and clinical presentation of the disease.

Mutation detection strategies for molecular screening in patients with MHE.

Wim Wuyts

Department of Medical Genetics University of Antwerp, Belgium.

Hereditary multiple exostoses (HME) is an autosomal dominant bone disorder characterized by the presence of bony outgrowths (exostoses) on the long bones. HME is a genetically heterogeneous condition and at present two causal genes have been identified: EXT1 on chromosome 8q23-q24 and EXT2 on chromosome 11p11.2. A third locus, EXT3 on chromosome 19p has been suggested but is controversial.

Since the identification of the HME causing genes, molecular analysis of HME patients has been optimized to increase sensitivity of the testing and reduce the cost. Recently, we further optimized the mutation screening protocol for both EXT1 and EXT2. For all coding exons DHPLC conditions were optimized and validated in a large set of HME patients with a known EXT1 or EXT2 mutation. All mutations could be detected under at least 1 DHPLC condition, providing a robust and sensitive alternative for labor extensive and more expensive sequencing analysis.

However, approximately 15 to 20% of HME patients does not show a mutation after extended sequence analysis of EXT1 and EXT2. We therefore expanded the screening protocol with FISH, MLPA and RNA analysis. This enabled us to detect (partial) EXT1 or EXT2 deletions in approximately 30% of EXT1/EXT2 mutation negative patients. At least one patient with two copies of both EXT genes showed loss of one EXT1 allele on the RNA level, but the underlying cause is still under investigation.

The various mutation detection strategies will be discussed as well as the mutation spectrum observed in HME patients.

Management of Multiple Hereditary Exostosis of The Axial (Hip And Spine) Skeleton

George H. Thompson, M.D.,

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Cleveland, OH 44106

Exostoses of the axial skeleton are uncommon lesions. Those involving the spine represent 4 to 7 percent of all primary benign spinal tumors. They can occur as solitary exostoses or in association with multiple hereditary exostoses (MHE). Pelvic exostoses, including those involving the proximal femur are also uncommon.

Spinal Lesions: We have recently evaluated our experience with spinal exostoses seen between 1972 and 2002. There were 12 patients, including 7 females and 5 males with a mean age at presentation of 24.2 years (range, 7-52 years). Five patients had MHE, while 7 had solitary exostoses. The mean age at presentation of the patients with MHE was younger at 16.8 years (range, 7-34 years) compared to 29.5 years (range, 22-52 years) for those with solitary lesions. Eight of the 12 patients had intraspinal lesions. These occurred most commonly in the cervical spine. All five patients with MHE had intraspinal exostoses. Three at C2 and one each in the thoracic spine and sacrum. The solitary intraspinal lesions occurred in the cervical (C2 and C6) or thoracic (T11). The most common chief complaint was pain (8 patients). Seven of these lesions resulted in symptoms consistent with spinal cord nerve root compression. Three patients with MHE and 4 with solitary exostoses had symptoms or findings consistent with neurological compression. Eight exostoses were treated surgically with eventual resolution of symptoms. The mean follow-up for patients treated surgically was 5.6 years (range, 0.5-13 years). Two patients had recurrence following resection of intraspinal lesions. Both had solitary lesions and underwent successful revision surgery. There was only one complication in the 8 patients treated operatively. This consisted of an anterior compartment syndrome following prolonged surgery for wide excision and stabilization of a thoracolumbar exostosis.

Pelvic Lesions: Pelvic lesions in MHE are relatively uncommon. When present, they usually involve the ilium and proximal femur but occasionally occur in the acetabulum, resulting in progressive subluxation of the femoral head. Lesions involving the proximal femur are usually a dysplasia rather than a true exostosis, but these too can become enlarged resulting in progressive hip subluxation. Excision of lesions about the pelvis include excision and possibly a proximally femoral osteotomy, if there is subluxation of the hip. An enlarging pelvic lesion in a skeletally mature individual is suggestive of malignant degeneration.

Conclusions: Exostoses involving the axial skeleton are relatively uncommon. Those involving the spine have a higher incidence of neurological symptoms due to spinal cord or nerve root compression. Any child with MHE presenting with neurological signs or symptoms should be evaluated by both computed tomography and magnetic resonance imaging. Without appropriate treatment, progressive neurological symptoms can occur. Lesions of the pelvis are also rare and a common site of malignant degeneration as an adult. The indications for treatment are pain, disfiguring mass, and progressive hip subluxation.

Session 2

**Thursday November 3, 2005
1:15pm - 5:00pm**

BONE DEVELOPMENT

Dan Wells, Chair

Fibroblast growth factor binding to perlecan isolated from the growth plate

Leigh West^a, Prasanthi Govindraj^a, Xiuqin Zhang^b, David M. Ornitz^b
and John R. Hassel^{a,c}

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Fibroblast growth factor (FGF)-2, FGF-9 and FGF-18 are three FGF's that have been shown to regulate chondrocyte proliferation in the growth plate. Heparin and heparan sulfate proteoglycans from endothelial cells have been shown to bind FGF-2. Perlecan is present in the growth plate as a proteoglycan containing both heparan and chondroitin sulfate chains and the phenotype of the perlecan knockout mouse shows it is necessary for the proliferation of chondrocytes in the growth plate. We evaluated the binding of FGF's to perlecan from the growth plate using a cationic filtration assay. We found FGF-2 bound primary to the heparan sulfate chains on perlecan but that the core protein was also involved in FGF-2 binding when the heparan sulfate chains were present. Removal of Chondroitin sulfate chains on perlecan enhanced FGF-2 binding slightly. FGF-1, FGF-9 and FGF-18 showed no binding to perlecan but FGF-7 showed a low level of binding. We also found, using recombinant FGF receptors in a separate capture assay that while the receptors would bind to a heparin-FGF-2 complex, the receptors would not bind to the perlecan-FGF-2 complex. Similarly, perlecan did not augment FGF-2 stimulation of [³H]-thymine incorporation in BaF3 cells. These data show perlecan can bind to FGF-2 by its heparan sulfate chains but that chondroitin sulfate chains on the perlecan acts to block transfer of the bound FGF-2 to the receptor.

FGFs that regulate growth plate development.

David M. Ornitz, Irene Hung, Zhonghao Liu,
Kory Lavine, Kai Yu, Hidemi Kanazawa, Ann Jacob.
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Washington University School of Medicine.

Fibroblast growth factors (FGFs) are essential molecules for mammalian development. A growing number of human genetic diseases that affect skeletal development are caused by point mutations in the genes encoding FGF receptors 1, 2 and 3. These disorders result in craniosynostosis and chondrodysplasia syndromes and thus demonstrate that FGF signaling pathways are essential regulators of chondrogenesis and osteogenesis. Loss of function and skeletal-specific conditional loss of function mutations in mouse FGF receptors 1-3 also show specific defects in skeletal development and in the structure and integrity of adult bone.

In contrast to the increasing amount of data demonstrating a role for FGFRs in skeletogenesis, there is very little information on the FGF ligands that signal to these receptors to regulate skeletal development, growth, remodeling and repair. Mice lacking FGF2 (bFGF) have a mild decrease in bone mass and trabecular bone formation, but no morphological defects in their skeleton.

Examination of skeletal development in mice lacking FGF18 revealed moderate skeletal dysmorphology and a significant delay in ossification of distal bones that is not seen in mice lacking FGF receptors 1, 2 or 3 in osteoblast or chondrocyte lineages. In contrast, analysis of mice lacking *Fgf9* revealed a delay in ossification primarily affecting proximal bones. The dysmorphology and delayed ossification phenotype of these knockout mice suggest that FGF9 and FGF18 signal to skeletal cells (chondrocytes and osteoblasts) to regulate early skeletal development and to non-skeletal mesenchymal cells to regulate peri-skeletal vasculogenesis and vascular invasion of the primitive growth plate.

We have also observed that mice lacking both FGF9 and FGF18 have very severe skeletal dysmorphology with delayed ossification and agenesis of the intramembranous bones of the cranium. These data further suggest that FGF9 and FGF18 form overlapping and inverted gradients in the appendicular skeleton that regulate both development and vascularization.

Syndecans: Cell Surface Modulators of Growth Plate Chondrocyte Behavior and Function

Maurizio Pacifici

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Syndecans are single-pass integral membrane components that serve as co-receptors for growth factors and cytokines and can elicit signal transduction via their cytoplasmic tails. We will present studies from our group on syndecan biology and function in the growth plates of developing long bones in chick and mouse embryos. Gain- and loss-of-function data indicate that syndecan-3 has important roles in restricting mitotic activity to the proliferative zone of growth plate and may do so in close cooperation and interaction with the signaling molecule *Indian hedgehog (Ihh)*, and that syndecan-2 may participate in growth plate-associated ossification. Biochemical and protein-modeling data suggest a dimeric/oligomeric configuration for syndecan-3 on the chondrocyte's cell surface. Analyses of embryos mis-expressing syndecan-3 or lacking *Ihh* provide further clues on syndecan-3/*Ihh* interdependence and interrelationships. The data and the conclusions reached provide insights into mechanisms fine-tuning chondrocyte proliferation and function and ossification events in the developing and growing skeleton and into how abnormalities in these fundamental mechanisms may subtend human congenital pathologies, including osteochondromas in hereditary multiple exostoses syndrome.

Delineation of Regulatory Networks Underlying BMP Action in Chondrogenesis

T. Michael Underhill¹, Lindsay M. Drysdale², Konstantina Karamboulas¹,
Matthew F. Cowan¹, Jian Wang³, William A. Horton⁴ and Lisa M. Hoffman²

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The BMP and GDF signaling pathways have well-established and essential roles within the developing skeleton in coordinating formation of cartilaginous anlagen. However, identification of *bona fide* targets that underlie the action of these signaling molecules in chondrogenesis has remained elusive. Using microarray-based methods coupled with functional profiling, we have identified the retinoic acid (RA) synthesis enzyme, *Aldh1a2*, as a principal target of BMP signaling—prochondrogenic BMPs or GDFs lead to attenuation of *Aldh1a2* expression and consequently, reduced activation of the retinoid signaling pathway. Consistent with this, antagonism of retinoid signaling phenocopies BMP4 action, whereas RA inhibits the chondrogenic stimulatory activity of BMP4. Further, moderate fold changes in endogenous retinoid signaling (< 4.5 fold) are sufficient to regulate expression of the chondroblast phenotype. In aggregate, these results establish a hierarchical relationship between the BMP and retinoid signaling pathways in chondrogenesis. These results will be presented along with additional findings that provide a molecular framework for understanding BMP action in the chondrogenic program. This research was supported by grants to T.M.U. from the Canadian Institutes of Health Research and the Canadian Arthritis Network.

How PTHrP regulates chondrocytes in the growth plate

Henry M. Kronenberg

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Chondrocytes in the growth plate are the engine that causes bone lengthening. Chondrocytes proliferate and secrete matrix. They then stop proliferating, enlarge several fold (hypertrophy), mineralize the matrix surrounding them, and then die. The matrix remaining provides a scaffold for subsequent formation of bone by osteoblasts. To assure a proper balance between chondrocyte proliferation and hypertrophy, elaborate regulatory mechanisms have evolved. Parathyroid hormone-related protein (PTHrP), a relative of the calcium-regulating hormone, parathyroid hormone, is secreted by perichondrial cells and chondrocytes at the ends of long bones of the limb. PTHrP acts to keep chondrocytes proliferating, thereby allowing the generation of more chondrocytes and delaying the onset of chondrocyte hypertrophy. Only when chondrocytes bearing receptors for PTHrP are sufficiently far from a source of PTHrP do the chondrocytes then stop proliferating.

PTHrP works by activating a receptor of the G protein-coupled receptor family. Activation stimulates the heterotrimeric G protein, G_s ; this activation leads to generation of cyclic AMP and activation of protein kinase A. These processes lead to suppression of the cell cycle inhibitor, p57, and also suppression of the expression of the transcription factor, Runx2. Suppression of p57 is a major mechanism that results in the continued proliferation of chondrocytes. Since Runx2 is a major inducer of chondrocyte hypertrophy, the suppression of Runx2 expression by PTHrP also contributes to the continued proliferation and delay of hypertrophy of chondrocytes.

As chondrocytes stop proliferating, they begin secreting Indian hedgehog. Indian hedgehog (Ihh) has multiple roles in regulating the growth plate. Ihh stimulates the synthesis of PTHrP, a role that then leads to further expansion of the layers of proliferating chondrocytes. Since these proliferating chondrocytes do not synthesize Ihh, the stimulation of PTHrP synthesis by Ihh serves to negatively regulate the expression of Ihh. Ihh also accelerates the conversion of round chondrocytes, found at the top of the growth plate, into flat columnar chondrocytes. Since the flat columns formed by these cells align in the primary axis of growth of the growth plate, this action of Ihh serves to help determine the final length and shape of the bones. Ihh also stimulates the proliferation of chondrocytes and acts on adjacent perichondrial cells to convert them to osteoblasts. Thus, Ihh is a master regulator of the growth plate which controls chondrocyte proliferation and differentiation, as well as the differentiation of adjacent osteoblasts.

Ihh signaling in the growth plate.

Andrea Vortkamp

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Endochondral ossification is a multistep process during which a cartilage template is successively replaced by bone tissue. Chondrocytes in the cartilage anlagen undergo several steps of differentiation until they become terminal hypertrophic and are subsequently replaced by bone. The secreted growth factor Indian hedgehog (Ihh) is expressed in a distinct population of chondrocytes that undergo hypertrophic differentiation. Ihh interacts with a second secreted molecule, Parathyroid Hormone related Protein (PTHrP), expressed in the distal ends of the cartilage elements in a negative feedback mechanism to regulate the onset of hypertrophic differentiation.

Analyzing a mouse line carrying a hypomorphic allele of *Ext1*, a glycosyltransferase necessary for the synthesis of heparan sulfates (HS), we have recently shown that HS negatively regulates the propagation of the Ihh signal in a concentration dependent manner. Our data strongly indicate that Ihh acts as a long range morphogen directly inducing the expression of PTHrP.

To further investigate the interaction between Ihh and PTHrP, we have started to analyze the role of the zinc finger transcription factor Gli3, which acts downstream of hedgehog signals in other organs. Ihh;Gli3 double mutants indicate that Gli3 acts as a repressor downstream of Ihh in regulating chondrocyte proliferation and the expression of PTHrP, and, thus, the onset of hypertrophic differentiation. Furthermore, our studies identified a new function of the Ihh/Gli3 system in negatively regulating the differentiation of distal, low proliferating (zone I) into central, high proliferating (zone II) chondrocytes. Whereas the domain of zone II chondrocytes is determined by the level of PTHrP, the transition of zone I into zone II chondrocytes is regulated by Gli3 independent of PTHrP.

Multiple Aspects of Ihh Signaling in the Endochondral Skeleton

Matthew Hilton

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Indian hedgehog (Ihh) is indispensable for proper endochondral bone development. In the developing cartilage, Ihh expressed in the prehypertrophic and early hypertrophic chondrocytes signals through chondrocytes and the perichondrium to regulate chondrocyte proliferation and hypertrophy. Previous studies have shown that Ihh on one hand directly regulates chondrocyte proliferation, and on the other hand indirectly controls chondrocyte maturation through regulation of Parathyroid hormone like hormone (Pthlh). However, it has not been understood 1) how Ihh transports through the cartilage, and 2) whether Ihh directly controls *pthlh* expression. Our recent studies with *Exostosin1* (*Ext1*) heterozygous mice, supports that heparan sulfate synthesis normally restricts the transportation of Ihh within the growth plate cartilage. More recently, through localized removal of *Smoothened* (*Smo*), the key mediator of hedgehog signaling, using a tamoxifen inducible Col2Cre transgenic mouse, we demonstrated that Ihh directly regulates *pthlh* expression in the immature articular chondrocytes. Additionally, we uncovered that Ihh may directly regulate the organization of columnar chondrocytes in a Pthlh-independent manner.

Session 3

**Friday November 4, 2005
9:00am - 11:15am**

EXOSTOSES/DEVELOPMENT

Jacqui Hecht, Chair

Differentiation-induced loss of heparan sulfate in human exostosis derived chondrocytes

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Robert J. Long⁴, Mary C. Farach-Carson⁴, Daniel D. Carson⁴

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An exostosis or osteochondroma is an aberrant bony growth occurring next to the growth plate either as an isolated growth abnormality or as part of the Hereditary Multiple Exostosis (HME) syndrome. Mutations in either exostosin 1 (EXT1) or exostosin 2 (EXT2) gene cause the HME syndrome and also some isolated osteochondromas. The EXT1 and EXT2 genes are glycosyltransferases that function as hetero-oligomers in the Golgi to add repeating glycosaminoglycans (GAGs) to heparan sulfate (HS) chains. Previously, we demonstrated that HS is markedly diminished in the exostosis cartilage cap and that the HS proteoglycan, perlecan, has an abnormal distribution in these caps. The present studies were undertaken to evaluate which chondrocyte-specific functions are associated with diminished HS synthesis in human chondrocytes harboring either EXT1 or EXT2 mutations. Systematic evaluation of exostosis cartilage caps and chondrocytes, both in vitro and in vivo, suggests that chondrocyte-specific cell functions account for diminished HS levels. In addition, we provide evidence that perichondrial cells give rise to chondrocytes that clonally expand and develop into an exostosis. Undifferentiated EXT chondrocytes synthesized amounts of HS similar to control chondrocytes; however, EXT chondrocytes displayed very poor survival in vitro under conditions that promote normal chondrocyte differentiation with high efficiency. Collectively, these observations suggest that loss of one copy of either the EXT1 or EXT2 gene product compromises the perichondrial chondrocytes' ability to differentiate normally and to survive in a differentiated state in vitro. In vivo, these compromised responses may lead to abnormal chondrocyte growth, perhaps from a perichondrial stem cell reserve.

**Analysis of exostoses in mice indicate signaling defects in chondrocytes
give rise to ectopic growth.**

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Hereditary Multiple Exostoses (HME) is an autosomal dominant disease characterized by osteochondromas on the ends of bones that form by endochondral ossification. The disease has been linked to mutations in EXT1 and EXT2, which encode subunits of the heparan sulfate copolymerase. To understand how a change in heparan sulfate biosynthesis might result in exostoses, null alleles of each gene have been created in mice. Homozygous null embryos arrest development at gastrulation, but heterozygous embryos appear normal, develop to maturity, and reproduce. They also exhibit occasional exostoses on the ribs and more rarely on other endochondral bones. EXT1 and EXT2 heterozygotes form exostoses at approximately the same frequency (14/101 and 20/120, respectively), whereas compound heterozygotes (EXT1^{+/-}EXT2^{+/-}) develop exostoses more frequently (60/165) consistent with the two genes acting through a common pathway. The exostoses arising in single and compound heterozygotes are indistinguishable by a number of criteria. Immunohistochemical and biochemical analyses revealed reduction of heparan sulfate in affected growth plates and in cultured chondrocytes, leading to shorter heparan sulfate chains. This in turn results in growth factor signaling defects in isolated chondrocytes. Exostoses appear to arise in perichondrial chondrocytes based on the histology of affected ribs and the appearance of exostoses in mice harboring a chondrocyte-specific inactivation of EXT1. Thus, we propose that signaling defects specifically in chondrocytes give rise to ectopic growth. The actual signaling pathway altered in heterozygous EXT animals will be discussed along with other phenotypes of mice altered in heparan sulfate biosynthesis.

Mice deficient in Ext2 lack heparan sulfate and develop exostoses

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Hereditary Multiple Exostoses (HME) is a genetically heterogeneous human disease characterized by the development of bony outgrowths near the ends of long bones. HME results from mutations in EXT1 and EXT2, genes that encode glycosyltransferases that synthesize heparan sulfate chains. To study the relationship of the disease to mutations in these genes, we generated Ext2-null mice by gene targeting. Homozygous mutant embryos developed normally until embryonic day 6.0, when they became growth-arrested and failed to gastrulate, pointing to the early essential role for heparan sulfate in developing embryos. Heterozygotes had a normal lifespan and were fertile, however, analysis of their skeletons showed that about 1/3 of the animals formed one or more ectopic bone growths (exostoses). Significantly, all of the mice showed multiple abnormalities in cartilage differentiation, including disorganization of chondrocytes in long bones and premature hypertrophy in costochondral cartilage. The finding that haploinsufficiency triggers abnormal cartilage differentiation gives insight into the complex molecular mechanisms underlying the development of exostoses.

Profiling of hereditary and solitary osteochondromas and peripheral chondrosarcomas on genomic, gene expression and protein level.

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Osteochondroma is a cartilage capped benign bony neoplasm on the outer surface of bones preformed by endochondral ossification. The cartilage cap of osteochondroma histologically resembles the morphological organization of an epiphyseal growth plate. A small percentage osteochondromas transform towards their malignant counter part, secondary peripheral chondrosarcoma. For Multiple Osteochondromas EXT1 located at 8q24 was shown to act as tumor suppressor gene in tumours of EXT1 mutant patients. We demonstrate that in hereditary tumors EXT expression is correlated with mutation status of the patient. Furthermore, EXT1, but not EXT2, is down-regulated in sporadic tumors, where 8q24 loss is a frequent phenomenon, but mutations or promoter hypermethylation are not found till now.

EXT genes are involved in biosynthesis of heparan sulphate proteoglycans (HSPGs). Immunohistochemical evaluation of a series (n=70) of both hereditary and solitary tumors revealed intracellular accumulation of HSPG core proteins and of shorter or less complex HS chains, in contrast to the extracellular expression of these molecules found in growth plates. HSPGs are involved in several growth signaling pathways, including in the negative feedback loop of the Indian Hedgehog (IHH) and parathyroid hormone-like hormone (PTHrP). PTHrP signaling is absent in osteochondromas, but reactivated upon malignant transformation towards chondrosarcoma. Disturbed expression of IHH signaling molecules has also been implicated in osteochondroma. With quantitative RT-PCR we were able to demonstrate similar expression levels of IHH signaling in osteochondromas as compared to the growth plate. A gradual decrease in expression of IHH signaling molecules was observed with increasing malignancy, suggesting that IHH signaling is inactivated and PTHrP signaling is IHH-independent in chondrosarcomas. cDNA expression profiling and immunohistochemical studies suggest that TGF- β -mediated proliferative signaling is upregulated in high-grade chondrosarcomas. TGF- β is a good candidate to regulate PTHrP signaling in high-grade tumors. Chondrosarcoma progression is further accompanied by down-regulation of energy metabolism-related genes and upregulation of the proto-oncogene *jun B*.

Dysplasia Epiphysealis Hemimelica (DEH, Trevor's disease) and metachondromatosis (MC) are considered in the differential diagnosis of solitary and hereditary osteochondromas. In a comparative study between DEH, MC and osteochondroma we were able to demonstrate that histologically DEH differs from conventional osteochondroma, while MC-related lesions do not. With qPCR we show that in contrast to osteochondroma, MC and DEH still express the EXT genes and immunohistochemical analysis showed that EXT downstream pathways are active. These results indicate that DEH and MC are indeed different entities and that so far unknown molecular defects in these lesions do not affect EXT signaling.

Session 4

Friday November 4, 2005

11:15am - 3:30pm

**EXT FUNCTION AND
NON-MAMMALIAN MODEL**

Scott Selleck, Chair

EXT1 and EXT2 proteins and heparan sulfate biosynthesis

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Heparan sulfate is a complex polysaccharide that plays an important role in several cellular processes, including normal fetal development, wound healing and inflammation. Defects in enzymes involved in heparan sulfate synthesis result in different abnormalities including abnormal skeletal and kidney development. Heparan sulfate is elongated by the alternating transfer of glucuronic acid (GlcA) and N-acetylglucosamine (GlcNAc) units. Concomitant with elongation, the polymer is modified through a series of reactions that requires the action of several different enzymes. The extent of these reactions varies, giving rise to heparan sulfate chains with different structural properties. The chain elongation reaction has been ascribed to a hetero-oligomeric complex of EXT1 and EXT2. Mutations in either EXT1 or EXT2 have been linked to the human disorder, hereditary multiple exostoses (HME), characterized by the formation of cartilage-capped bony outgrowths at the end of the long bones.

The individual functions of EXT1 and EXT2 in heparan sulfate chain elongation are currently unknown. EXT1 alone has the capacity to elongate heparan sulfate chains *in vitro*. Furthermore, reduced EXT1 expression levels results in the formation of heparan sulfate chains that are shorter than those normally synthesized. The level of EXT2 protein modifies the catalytic properties of EXT1 but the role of EXT2 in heparan sulfate chain elongation is not clear.

To evaluate the effect of EXT2-mutations on heparan sulfate structure, we have generated transgenic mice with a general and constitutive tissue expression of wild-type or mutated EXT2. To understand the individual roles of EXT1 and EXT2, we have overexpressed the proteins or reduced their levels in mammalian cell systems and studied the effects of these manipulations on heparan sulfate structure.

**Heparan sulfate biosynthesis and sulfation:
Exploring function in synapse assembly and vascular development**

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Heparan sulfate is a structurally diverse molecule that is important for both the control of cellular responses to secreted growth factors, and controlling their distributions in the matrix. Our group employs a number of model organisms and systems to understand the functions of heparan sulfate-modified proteoglycans during development. HSPGs have long been known to be present at the neuromuscular junction in vertebrates, but the function of this class of molecules has not been explored at this well studied synapse. We have used mutations affecting the proteins responsible for HS chain initiation (*brother of tout-velu*, *botv*, an ortholog of and *Ext1*), chain polymerization (*tout-velu*, *ttv*; *sister-of-tout-velu*, *sotv*, orthologs of vertebrate *Ext* genes) and sulfation (*sulfateless*, *sfl*; ortholog of *N-deacetylase N-sulfotransferase*) in the fruitfly *Drosophila* to assess the role of HS in neuromuscular development and function. We find that animals defective for the HS biosynthetic and modifying enzymes show defects in synaptic transmission. Compensation to presynaptic defects remains however, with increases in muscle responses and elevated levels of glutamate receptors in the postsynaptic cell.

Our studies of the requirement for HS in vascular development employ the zebrafish *Danio rerio*. Using both mutants affecting *Ext2* and *Extl3* (*boxer* and *dackel*, respectively), as well as morpholinos directed against the mRNAs from these genes, we have found that HS synthesis is required for normal angiogenesis in zebrafish embryos. We have also established methods for identifying small molecule inhibitors of HS synthesis and modification using human tissue culture cells. A small molecules derived from this screen affects HS biosynthesis in zebrafish embryos and likewise disrupts normal vascular development.

Embryonic Stem Cells can be used to investigate the role of Heparan Sulphate in Development and Disease

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For several years now, work using model organisms has demonstrated that heparan sulphate (HS) in association with specific core proteins is a downstream effector of several regulatory molecules that modulate changes in the morphology, mobility and proliferation of developing cells. The genetic analysis of inherited diseases such as HME has revealed the importance of HS in cell growth regulation and tissue-specific patterning during human development. In addition to our on-going study of the role of HS at the molecular level, we have established the novel approach of using murine and human embryonic stem (ES) cells to provide *in vitro* model systems for the study of the developmental biology of HS. ES cells undergo symmetrical self-renewal in culture whilst retaining the ability to differentiate into all foetal and adult lineages. There are three alternative fates for ES cells; they can remain pluripotent, they can differentiate or they can undergo apoptosis. The signalling molecules that control this decision process are complex, and consist of a subtle interplay of secreted factors, cell-autonomous factors and cell-adhesion molecules. Many of these signalling proteins are familiar to the proteoglycan (PG) field, being HS-dependent growth factors, morphogens or matrix-resident PGs secreted by the ES cells, or by the fibroblasts used as a feeder layer. ES cells therefore present an experimentally tractable *in vitro* system in which the role of HS in multiple interacting signalling processes can be assessed. The major benefit of the system is that we can monitor cells as they transform from a pluripotent phenotype to differentiated lineages. This enables us to study the relationship between developmentally-regulated expression of HS-biosynthetic enzymes (such as EXT-1) and the structural and functional attributes of HS. This has become a hotly debated issue in the field as evidence has emerged concerning the deleterious effects of mutations in these enzymes on embryogenesis and the functions of HS in the adult. We are currently using an *ext1* knock-out mouse ES cell line (from Prof. Esko, UCSD) to detail the role of cell-surface HS in the earliest stages of differentiation and lineage commitment. This work will enable us to better understand the function of HS in co-ordinating the multiple interacting pathways influencing cell development and differentiation in the normal and disease state.

Developmental regulation of heparan sulfate proteoglycan synthesis during *Drosophila* embryogenesis.

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Signaling by the BMP4 homolog Decapentaplegic (Dpp) is critical for cell fate specification in a wide variety of tissues and at several stages during *Drosophila* development. The most extensively studied roles for Dpp are in patterning the dorsal region of the early embryo and in regulating cell proliferation and patterning in the wing imaginal discs. Dpp activity in the wing disc requires heparan sulfate proteoglycans (HSPGs), and mutations in *tout velou* (ttv) and sister of *tout velou* (sotv) that encode the GAG chain polymerases, impair Dpp signaling. Ttv and Sotv activities are also essential for Hedgehog (Hh) and Wingless (Wg) signaling, both in the imaginal discs as well as in the embryo. Surprisingly however, embryos lacking ttv, sotv and other HSPG biosynthetic enzymes do not show any alterations in dorsal patterning, a phenotype characteristic of disruptions in the Dpp pathway. Thus our data suggested that the Dpp pathway, in contrast to the Hh and Wg pathways, appears to be differentially sensitive to loss of HSPGs at different developmental stages.

In order to understand the basis for these observations we examined the temporal regulation of GAG chain addition to HSPG core proteins. We found that GAG chain synthesis is under tight developmental control. Essentially no synthetic activity is detectable in the first three hours of embryogenesis with a rapid onset between three and four hours following fertilization. The early time period when the biosynthetic process is inactive correlates precisely with the interval during which a Dpp/BMP activity gradient is established, while the onset of GAG chain addition coincides with the time when Hh and Wg signaling first become active in patterning the embryonic epidermis. We find that the timing of GAG chain addition is not controlled by the regulated expression or activity of a pathway component. Instead our data argue that GAG chain synthesis is controlled at a post-transcriptional level through regulated translation of at least one of the GAG chain polymerases. Interestingly, this regulatory mechanism appears to be phylogenetically conserved, suggesting that stage or tissue-specific regulation of GAG chain addition could represent an important strategy in altering the sensitivity of specific signaling pathways at unique stages during development.

Zebrafish as a model for studies on the hereditary multiple exostosis.

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Zebrafish is an easy to maintain, small tropical fish with transparent embryos that develop outside the mother's body. Their short generation time (3-4 months), high fertility rate (hundreds of eggs per week), rapid development (most organs develop within first 48 hours post fertilization) and advanced genetic techniques (transgenics, forward and reverse genetics) make them an outstanding model for biomedical research. Furthermore, development of their cartilaginous skeleton occurs by similar mechanisms to that of humans, which means zebrafish are suitable as a model for studies on human skeletal diseases. Using forward genetic screens, hundreds of zebrafish mutations that affect cartilage morphogenesis and/or differentiation have been identified. We positionally cloned two mutations in exostosin genes: *dackel* (*dak/ext2*) and *boxer* (*box/extl3*) (Lee *et al.*, 2004, Neuron. 44: 947-960) and are currently using these to study the development of exostoses. Homozygous *dak/ext2* mutants show a similar disorganization of cartilaginous skeleton to that seen in HME tumors i.e. chondrocytes, instead of forming long stacks of flattened cells, form non-polarized clusters of rounded cells. Cartilage formation and differentiation remains unaffected in *dak/ext2* mutants, which suggest that the *dak/ext2* phenotype probably results from changes in cell division planes or/and cell movements. In support, electron microscope observations verified the presence of abnormalities in the cytoskeleton of *dak/ext2* mutant chondrocytes. Furthermore, malformations of cartilage similar to those seen in *dak/ext2* are also present in another zebrafish mutant called *pipetail* (*ppt/wnt5a*) that is involved in the non-canonical Wnt/Ca²⁺ planar polarity pathway. Interestingly, both *dak* and *ppt* mutants show a significant delay in endochondrial ossification whereas membranous bones are formed normally. The similarities between these two mutants suggest *Ext2* may act through non-canonical Wnt signaling.

In addition to the exostoses-like phenotype, *dak/ext2* and *box/extl3* also share other developmental defects (missorted retinotectal projections and malformed pectoral fins) and both of these mutants have significantly reduced level of heparan sulfate proteoglycans (HSPGs). A third mutant, called *pinscher* (*pic*), also has the mentioned above defects, suggesting that *pic* is in the same genetic pathway. We have recently positionally cloned *pic* and shown it does not belong to the exostosin gene family. This strengthens the possibility that non-*Ext1/Ext2*-related cases of HME might be due to mutations in other genes involved in the synthesis of HSPGs.

This work is supported by Wellcome Trust and Cancer Research UK.

Session 5

**Friday November 4, 2005
3:30pm - 5:00pm**

NON-BONE PHENOTYPES

Yu Yamaguchi, Chair

**Phenotypes of conditional Ext1 knockout mice:
Insights into non-skeletal symptoms of MHE**

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Heparan sulfate proteoglycans have been implicated in various cell biological and developmental processes, such as growth factor and morphogen signaling, cell adhesion and migration, and extracellular matrix assembly. To study the physiological roles of heparan sulfate proteoglycans in the mammalian development, we created conditional allele of Ext1, the gene encoding a glycosyltransferase required for heparan sulfate biosynthesis. Mice carrying this allele have been crossed with several Cre transgenic mice to determine the function of heparan sulfate in different embryonic and adult tissues. I will present our recent findings on the phenotypes of these conditional knockout mice, and discuss molecular mechanisms underlying such phenotypes and potential implications into non-skeletal symptoms of MHE.

Structure and Biosynthesis of Mouse Brain Heparan Sulphate.

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Heparan sulfate (HS) biosynthesis involves the action of a complex set of enzymes with polymerase (EXT), epimerase and sulfotransferase (ST) activities. Multiple isoforms of N- and O-STs decorate the nascent HS chains with specific sulfation patterns which confer selective biological functions. We have been studying HS structure and biosynthesis in model organisms such as mice and nematode worms since they provide opportunities to study the expression of these enzymes in relation to the structure and activities of the HS produced. In previous studies in mice we found that there are stage-specific combinations and distinct spatiotemporal expression patterns of HSST isozymes that underlie the synthesis of different HS species in developing brain. This data indicated that differential HS biosynthesis results in the synthesis of structurally variant HS species which form functional signaling complexes with growth factors essential for normal brain development. Regulated synthesis and the levels of specific HS species could be a mechanism for regulation of proliferation and differentiation in the developing brain. In recent studies we have become interested in the possibility that the levels or structures of HS may be altered in the brain tissues of mice heterozygous for the EXT1 gene, and that biochemical defects of this type could underly nervous system abnormalities observed in humans with this genotype. We have purified HS from normal and EXT1 +/- mice and are currently performing detailed structural analyses on these samples. Data will be presented to address the question as to whether there are alterations in the amounts and/or structures of HS produced in the brains of EXT1 +/- mice.

Keloid Formation Following Surgical Treatment of Multiple Hereditary Exostoses

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Introduction: Multiple hereditary exostoses (MHE) is an autosomal dominant trait characterized by numerous cartilage capped tumors in areas of actively growing bone. The formation of keloids following surgery for MHE has not previously been described.

Methods: A retrospective case-controlled study was undertaken to test the hypothesis that patients with MHE are at higher risk for keloid formation following excision of an exostosis. The study population consisted of a study group of 25 children and adolescent cases of MHE randomly selected from a tumor database at our institution and a control group of 25 age-matched cases of solitary exostosis (osteochondroma). All patients participated in a phone interview that consisted of questions regarding the number of surgeries, recurrence of lesions, wound healing problems, keloid formation, keloid site and dimensions, and any revision surgery. All patients with wound healing problems or suspected keloids were asked to take clinical pictures and mail them in. Based on clinical criteria these cases were identified as keloids or non-keloids.

Results: 83 surgeries were performed in 25 patients with MHE for primary excision of their exostoses. 25 surgeries were performed in 25 cases of solitary exostoses. 12 keloids formed in 7 patients in the MHE study group. No patients who underwent excision of solitary exostoses formed keloids. Diagnosis of MHE was a statistically significant risk factor for formation of keloids following surgery ($p < .05$). Maximal keloid width ranged from 5-10cm. Scar revision was performed in four of the seven children with keloid formation with MHE, of whom two required additional scar revision procedures.

Discussion: Wound healing problems in MHE, in particular a tendency for keloid formation, have not previously been reported. Our retrospective, case-controlled study demonstrated a significant correlation between keloid formation and surgery for MHE. The risk for keloid formation should be discussed with patients as part of informed consent prior to surgery for removal of exostoses in MHE.

Session 6

**Saturday November 5, 2005
9:00am - 11:30am**

HME-RELATED DISEASES

Benjamin Alman, Chair

P53 and Rb in Cartilage Tumours in Mice

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Chondrosarcomas can arise from being cartilage lesions, such as enchondromas and osteochondromas. In enchondromatosis, there is a high rate of malignant change, reported to be as high as 50% in cases of Maffucci syndrome. Cytogenetic and mutational analysis studies identified mutations or deletions in p53 or Rb in roughly one third of chondrosarcomas. As such, we examined the role of these tumor suppressor genes using a mouse model of enchondromatosis. We crossed *p53* and *Rb* knockout mice with mice overexpressing *Gli2* driven by the type II collagen regulatory elements. The *Gli2* transgenic mice develop cartilaginous rests in their metaphysis, similar in appearance to enchondromatosis. Embryonic limbs from *Gli2;p53*^{-/-} and *Gli2;Rb*^{-/-} were compared to wild type littermates, and postnatal *Gli2;p53*^{+/-} and *Gli2;Rb*^{+/-} mice were examined at two month intervals until 8 months of age, and their phenotype compared with wild-type littermates. Mice were sacrificed and limbs analyzed using histology, Safranin-O staining, type X collagen immunohistochemistry, proliferation rate and apoptosis rate. Larger, hypercellular, cartilaginous lesions containing pleomorphic cells arose in the *Gli2;p53*^{+/-}, at an increasing incidence starting at 2 months of age. By 8 months, 75% of these mice developed these larger lesions. This was associated with an increase in cell proliferation. *Gli2;Rb*^{+/-} mice also developed these larger lesions, but only at 8 months of age. Examination of the fetal limbs showed an expanded growth plate, involving all zones in the *Gli2;p53*^{-/-} mice, compared to the other genotypes. P53 deficiency modulates the effect of overexpression of *Gli2* in chondrocytes, resulting in a change in the growth plate and the development of larger, hypercellular cartilage lesions, perhaps by increasing the number of chondrocyte cells in the growth plate. This data also suggests that tumor suppressor genes play a role in cartilaginous neoplasia.

Tibia Dyschondroplasia: An Example of Defective Hedgehog Signaling?

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Tibial Dyschondroplasia (TD) is a skeletal disease common to rapidly-growing young avians. This condition is characterized as a mass of avascular cartilage in the epiphyseal growth plate of tibiotarsus and tarsalmetatarsus. The lesion occurs spontaneously with an incidence ranging from 10-90%. Exposure to dithiocarbamates induces a high incidence of the lesion without impairing growth rate. The lesion appears to initiate in the prehypertrophic zone adjacent to the perichondrium, resulting in a triangular lesion of variable size. The chondrocytes in the lesion fail to achieve complete hypertrophy and undergo apoptosis. This latter characteristic has severely impaired attempts to identify the metabolic defect responsible for this skeletal disease. For a number of years, we have been pursuing the hypothesis that TD occurs as a result of perturbation in Indian Hedgehog (Ihh) signaling. This is based on the observation that Ihh, which plays a key role in skeletal physiology, is localized in the zone where the lesion appears to be initiated. Our first approach was to confirm this hypothesis by studying Ihh expression in cultured chondrocytes. This approach was abandoned due to the extreme cytotoxicity of the dithiocarbamates. Since sterolization is an important post-translational modification of hedgehog proteins, an inhibitor of cholesterol biosynthesis (a statin) was fed to young chicks as a means of perturbing hedgehog activity. These chicks exhibited depressed growth rate and a TD-like lesion in the prehypertrophic zone of the epiphyseal growth plate. Supplementary dietary cholesterol resulted in normal growth plate morphology, although the growth rate depression was not reversed. These results were exciting, but we were faced with a dilemma: which hedgehog is being inhibited? Wu *et al.* (2002) have reported that both Ihh and Sonic Hedgehog (Shh) are expressed in the post-natal avian growth plate, with Shh being expressed distal to Ihh. We have confirmed the presence of Shh in lysates of hypertrophic chondrocytes with western blotting. The expression of two hedgehogs at different locations in the growth plate supports previous suggestions that hedgehog proteins have dual actions in growth plate physiology. Currently we are using immunohistochemistry, western blotting and microarray technology to identify the downstream targets of hedgehog genes responsible for the impaired angiogenesis associated with the development of tibial dyschondroplasia.

Microarray Analysis Reveals Malignancy-Related Markers in Human Chondrosarcoma

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Introduction: Aggressive chondrosarcomas can be difficult to distinguish histologically from less aggressive malignant tumors or from benign enchondromas, due in part to the retention of cartilage-like phenotype. To identify markers of malignancy in chondrosarcomas, we have performed a comparative transcriptome study of chondrocytes isolated from normal cartilages, enchondromas and chondrosarcomas of different grades (grade 1 and grade 3) using Affymetrix Human 133A microarrays. These studies revealed a number of genes that were differentially expressed in high-grade chondrosarcomas. These results suggest a number of potentially useful markers of tumor progression in chondrosarcoma.

Essential Results: Microarrays established a list of genes that were differentially expressed in high grade tumors including thrombospondin-1 (THBS1), decorin, TIMP-3, MGMT (down-regulated), TRAG-3 and FGF-5 (up-regulated). Among these differentially expressed mRNAs, Maspin (Serpine B5) was strongly up-regulated in high grade chondrosarcomas (Table 1). The microarray results were validated at the RNA level by semi-quantitative RT-PCR, on RNA from a collection of 10 different chondrosarcomas of various grades. This analysis showed increased Maspin mRNA in each case compared to normal chondrocytes. However, high grade cells consistently showed the highest expression levels (data not shown). Western blots for Maspin confirmed that this protein was easily detectable in high grade chondrosarcoma cell extracts but was virtually absent from normal cells, benign cells, and low-grade chondrosarcoma cells (Figure). Additional western blots confirmed microarray results that indicated down regulation of MGMT in high grade chondrosarcomas (data not shown).

Discussion: Our findings show a number of changes in gene expression that distinguish high-grade chondrosarcoma from low-grade chondrosarcoma, benign enchondroma, and normal chondrocytes. These results were verified by rtPCR or western blots, indicating that the microarray results were a valid measure of mRNA levels for these genes. We found that Maspin was among the most strongly up-regulated genes and western blotting confirmed that this protein is markedly over-expressed in high-grade cells. Taken together these results show that Maspin can be a reliable marker of tumor progression in chondrosarcoma. In addition, many of the genes that were found to be differentially regulated in high-grade cells are known to be regulated by promoter methylation. The involvement of such an epigenetic mechanism in the transcriptional regulation of those genes is currently under investigation.

References

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Enchondromatosis

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Enchondromatosis is the term for a collection of syndromes with overlapping clinical features and is characterized by the presence of multiple enchondromas (1). Enchondromas are benign cartilage producing tumors located in the medulla of bone. Most often they can be found in the long bones, especially of hands and feet. In about 25% of enchondromatosis patients malignant transformation of an enchondroma into a conventional central chondrosarcoma occurs compared to <1% in patients with a solitary enchondroma (1;2). Signs for malignant transformation are sudden growth, fatigue and pain in the affected area (3). Pathologic fractures at the side of an enchondroma can occur, not necessarily pointing to malignant transformation.

The malignant transformation of enchondromas into central chondrosarcomas can result in an adverse prognosis for the patient, in parallel to malignant transformation of osteochondromas into peripheral chondrosarcomas. Both central and peripheral chondrosarcomas share histological similarities and therefore they are graded in the same way. Following the grading system of Evans *et al* (4) – the most widely used grading system for chondrosarcomas-, three grades of malignancy are discerned, grade I, grade II and grade III. Grading is currently the best prognostic indicator. Where as in grade I chondrosarcomas seldom metastasize, 10-33% of grade II and ~70% of grade III chondrosarcomas metastasize (4;5).

The two most frequent enchondromatosis syndromes are Ollier disease (6;7) and Maffucci syndrome (8;9). Both have multiple enchondromas but in Maffucci syndrome this is combined with multiple haemangiomas and/or lymphangiomas (8). These are rare non-hereditary syndromes, and in a group of patients unilateral predominance has been described. Skeletal deformations become apparent after birth. Almost all patients with enchondromatosis have orthopedic complications, of which short stature is the most prominent (10;11). Additionally, deformities of the tubular bones resulting in leg-length discrepancy are described (10;11).

Besides Ollier disease and Maffucci other, even more rare, syndromes have described, which have been subdivided based on the involvement of hands and feet, spine and hereditary (12;13). For instance, spondyloenchondromatosis is characterized by involvement of spinal cord, mild involvement of hands and feet and a distribution in a autosomal dominant fashion (12;14). Generalized enchondromatosis is described to have severe involvement of the hands and feet, mild involvement of the spinal cord and a distribution in a autosomal recessive fashion (15). One other interesting subclass is metachondromatosis. This is characterized by the combined presence of multiple enchondromas and exostoses, which are in contrast to those in Multiple Osteochondromas (16) pointed toward the nearby joint. In these patients there is no evidence of spinal involvement and the distribution follows an autosomal dominant pattern (17;18)

ABC's of MHE WORKSHOP

**Saturday November 5, 2005
1:00pm – 5:00pm**

Sarah Ziegler, Chair



The ABC's of MHE: Everything you need to know about Multiple Hereditary Exostoses workshop.

Saturday Nov 5, 2005

Speakers

- ❖ **Jeffrey Esko, Ph.D.,[#] Scott Selleck, M.D., Ph.D.,⁺ Presenting an overview of the scientific sessions of the conference.**
[#] University of California-San Diego, Department of Cellular Molecular Medicine, Director, Glycobiology Research and Training Center, San Diego, CA.,
⁺ University of Minnesota Department of Pediatrics, Genetics, Cell Biology and Development, Minneapolis, MN.
- ❖ **Wim Wuyts, Ph.D., Presenting the ABC's of MHE – Genetics.**
Supervisor, DNA Diagnostics, Department of Medical Genetics, University of Antwerp, Belgium.
- ❖ **Jacqueline Hecht, Ph.D.,[#] Sandra Darilek M.S.,⁺ Presenting study results of "Hereditary Multiple Exostoses and Pain".**
[#] Medical Genetics, Family Studies, University of Texas Houston Medical Center and Medical School, Department of Pediatrics, Division of Medical Genetics, Director, Genetic Counseling Program, Houston, TX., ⁺ Genetic Counselor.
- ❖ **Ashish Sinha, M.D., Ph.D., DABA., Presenting the issue of chronic pain and the need for treatment.** Department of Anesthesiology & Critical Care School of Medicine, University of Pennsylvania Philadelphia PA.
- ❖ **Harish Hosalkar, M.D.,[#] John P. Dormans, M.D.,⁺ Presenting The ABC's of MHE**
[#] Orthopaedic Resident, The Children's Hospital of Philadelphia,
⁺ Chief of Orthopaedic Surgery, The Children's Hospital of Philadelphia, Professor of Orthopaedic Surgery University of Pennsylvania School of Medicine.
- ❖ **John E. Herzenberg, M.D., FRCSC, Presenting the use of Fixators.**
Chief of Pediatric Orthopedics, Sinai Hospital; Co-Director, International Center for Limb Lengthening Rubin Institute for Advanced Orthopaedics Baltimore, MD.

Genetic Aspects of MHE.

Wim Wuyts

Department of Medical Genetics University of Antwerp, Belgium.

MHE is a genetic disorder and therefore it not only affects the patient and his/her relatives, but also future generations. Genetic counseling of MHE patients and their family is therefore an important aspect and should be offered to all MHE patients. However, to understand the genetics of MHE one should have an understanding of basic genetics. In this workshop commonly used genetic terms and mechanisms will be explained and the specific genetic aspects of MHE will be discussed in detail. An overview will be presented of the options and difficulties associated with genetic screening for MHE.

Hereditary Multiple Exostosis and Pain

Jacqueline Hecht¹ and Sandra Darilek²

¹Department of Pediatrics, University of Texas Houston Medical Center

²Genetic Counselor Baylor College of Medicine

This study was undertaken to characterize pain in individuals with hereditary multiple exostosis (HME). Two hundred ninety-three patients with HME completed a questionnaire designed to assess pain as well as its impact on their life. Eighty-four percent of participants reported having pain, indicating that pain is a real problem in HME. Of those with pain, 55.1% had generalized pain. Two factors were found to be associated with pain outcome: HME-related complications and surgery. Individuals who had HME-related complications were five times more likely to have pain, while those who had surgery were 3.8 times more likely to have pain. No differences were found between males and females with respect to pain, surgery, or HME-related complications. The results of this study indicate that the number of individuals with HME who have pain has been underestimated and that pain is a problem that must be addressed when caring for individuals with HME.

Pediatric Pain Management

Ashish Sinha
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Pain in the pediatric patients is frequently under treated due to a variety of factors. From under recognition of the pain itself to myths that children do not feel as much pain as adults, because of immature peripheral and central nervous systems. Part of the problem is the mistaken belief that pain is less harmful than the side effects of analgesic therapy. Lack of awareness of treatment options and ignorance of analgesic pharmacology in children compound this problem.

Pain assessment in children has been mandated by JCAHO and frequently referred to as the 5th vital sign. The need for accurate pain assessment is essential for accurate pain management. Sometimes this is an approximation because of limited verbal communication in the younger children population. Multiple reasons contribute to the denial of pain by children. Useful tools for pediatric pain assessment, depending on the age of the child, include CRIES, FLACC, Wong Baker faces scale and VAS (Visual Analog Scale) scores.

Treatment of chronic pain is handled differently than that of acute pain in children. Pain treatment in the setting of chronic pain in children is multimodal as in adults and has components of depression that should be addressed appropriately. The concept of the WHO pain ladder being applied to children is consistent with appropriate and responsible pain management. Pain that arises in the musculoskeletal system has issues with reluctant limb usage with its attendant problems, of dystrophy and atrophy. An expert in physical therapy should be involved in the handling of these issues.

The ABC's of MHE

Harish Hosalkar,[#] John P. Dormans,⁺

[#]Orthopaedic Resident, The Children's Hospital of Philadelphia

⁺Chief of Orthopaedic Surgery, The Children's Hospital of Philadelphia

Professor of Orthopaedic Surgery, University of Pennsylvania School of Medicine

Multiple hereditary exostosis (MHE) is an inherited disease causing the development of numerous cartilaginous exostoses throughout the skeleton. It is most commonly inherited as an autosomal dominant loss of function mutation of either the *EXT1* or *EXT2* genes with almost complete penetrance. Common problems for children with MHE are pain and tenderness due to compression of tendons and nerves by the exostoses, skeletal deformity due to altered growth of long bones, cosmetic concerns, and rarely ischemic complication due to compression of vascular structures. As a result, most children with MHE will undergo several procedures for removal of painful or deforming lesions.

The ABC'S of MHE is a patient and parent-friendly manual that outlines the common skeletal manifestations of MHE. This extensive review addresses the diagnostic tools including important features on clinical exam, characterization of lesions, diagnostic work up including imaging features and histology. We have attempted to outline the established patterns of involvement of MHE in various parts of the body i.e. mainly the skeletal system and their possible treatment options. A specific note is made in each subsection regarding what the parents should watch out for. Finally a glossary of procedures and terminology is presented.

External Fixation and Stapling for Angular Deformities and Limb Length Discrepancies in Multiple Hereditary Exostosis (MHE)

John E. Herzenberg, MD, FRCSC

Head of Pediatric Orthopaedics, Sinai Hospital of Baltimore
Co-Director, International Center for Limb Lengthening
Rubin Institute for Advanced Orthopedics
Baltimore, MD 21215

General considerations: MHE causes valgus in the knee and ankle. The cause is tethering of the growth plates, leading to asymmetric growth and limb length discrepancy. Treatment of valgus (knock knee) deformity improves awkward gait, and prevents abnormal joint loading that can cause premature knee arthritis. Treating leg length discrepancy improves gait mechanics and prevents low back pain caused by pelvic tilt. Mild angulations in growing children can be treated with hemi-epiphyseal stapling using Blount staples (Zimmer) in pre-teens, or the new 8-plate (Orthofix) in children as young as 3 years (where staples might dislodge). For children near skeletal maturity, and for adults, osteotomy and gradual correction with external fixators is the most accurate way to correct angulation and length problems. The TSF (Smith & Nephew) and MAC (EBI) enable multiplanar correction with simultaneous lengthening. For adults without angulation, there is an implantable telescopic lengthening nail called the ISKD (Orthofix) which lengthens the femur or tibia without an external fixator.

Valgus knee: Assess angulation on a long standing x-ray that includes hip, knee and ankle on a single cassette and a long lateral film. Measure the mechanical axis deviation (MAD), lateral distal femoral angle (LDFA), medial proximal tibial angle (MPTA), posterior proximal tibial angle (PPTA) and posterior distal femoral angle (PDFA). These tests usually localize the problem to the proximal tibia. Consider stapling for young patients with sufficient growth. Older children are treated with gradual correction using an external fixator and corticotomy. Consider simultaneous peroneal nerve decompression and resection of the fibular head osteochondroma.

Valgus ankle: Assess angulation on standing films centered on the ankle. Measure the lateral distal tibial angle (LDTA) and anterior distal tibial angle (ADTA). Evaluate for presence of compensatory subtalar contractures. Staple the medial distal tibia for younger patients without subtalar contractures. If there is an established subtalar contracture that makes the foot plantigrade, then the valgus tilt of the ankle might best be left untreated.

Leg length discrepancy: For discrepancies under 2 cm, use shoe lifts. In skeletally immature patients, consider epiphyseodesis of the long leg. Lengthening the short leg is preferred if there is residual angular deformity to be corrected. Predicting adult height and limb length discrepancy with the Multiplier method helps families to decide which option to choose. In mature patients, without angulation, lengthen either with ISKD or lengthening over nail (LON) methods to eliminate or decrease external fixation time. Shortening the long leg in adults by up to 4 cm can be safely done in the femur over an intramedullary nail as an alternative to lengthening.

Forearm problems: For the short ulna without angulation in young children, lengthen the ulna with an external fixator to prevent the secondary changes of distal radius ulnar deviation and radial head dislocation. Older children need more complex treatment: ulnar lengthening with distal radio-ulnar fixation to gradually reduce the dislocated radial head, followed by staged distal radial osteotomy for angular correction. Mild ulnar deviation of the distal radius may be amenable to hemi-epiphyseal stapling techniques.

The American Association of Multiple Enchondroma Diseases



Founded in 1998, incorporated in 2002 as a not-for-profit corporation and registered as a tax exempt organization with the US Internal Revenue Service, AAMED is comprised of individuals with Ollier's disease, Maffucci's syndrome, enchondromatosis, their families, and physicians.

AAMED is THE source for news and information about bone tumor diseases, research and services for adults and children with Enchondromatosis, Multiple Enchondroma, Ollier's disease, Maffucci's syndrome, and their families.

Board of Directors:

President, Ellen Bregg

CEO, Director of Research, Susan Challen

Research Coordinator, Sarah Ziegler

AAMED: www.AAMED.NET



Houston Shriners Hospital

Family-Centered Care

Recognizing that the family plays a vital role in a child's ability to overcome an illness or injury, the Houston Hospital helps the family provide the support the child needs by involving the family in all aspects of the child's care and recovery. The purpose of all Shriners Hospitals is to provide care to orthopaedically disabled and burned children to help them lead fuller, more productive lives. By promoting the importance of the family and helping it become a stronger support system for the child, the Houston Hospital can accomplish its purpose more effectively.

Vision

Ours will be a place where patients and families easily access our services, partner with us in care, and acquire the skills towards transitioning into a productive adult life. All of this is based on education and research which provide the foundation of our care for now and in the future.

Mission

To provide the quality of pediatric, orthopaedic care necessary to allow our children the opportunity to be productive and involved members of their community.

To provide for the education of physicians, other health care professionals, patients, families and the fraternity community-wide.

To engage in clinical research with a focus on patient outcomes assessment.

Research

More than 300 different and distinct diseases affect the bones, joints and supporting structures, composed mainly of fibrous tissues such as ligaments, tendons and cartilage. Many of these diseases are congenital, others are inherited, and a great many are both congenital and inherited. However, very few of these diseases have identifiable causes.

Shriners Hospitals have been involved in clinical children's orthopaedic research since the early 1920s, and in the early 1960s, Shriners Hospitals aggressively entered the structured basic research field. Advances in research are shared widely among the 22 Shriners Hospitals and with other hospitals throughout the country. The Houston Hospital conducts clinical and basic research in the following areas:

- ✚ Clinical research studies in gait analysis
- ✚ Genetic linkage studies of club foot
- ✚ Genetic and behavioral studies of spina bifida
- ✚ Genetic studies of pseudoachondroplasia, a form of dwarfism
- ✚ **Molecular studies of hereditary multiple exostosis, a condition marked by "spurs" or bony outgrowths on bone**
- ✚ Regulatory and structural studies of cartilage oligomeric matrix protein (COMP), a key component in the growth and formation of cartilage

<http://www.shrinershq.org/shc/houston/index.html>

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