The Sixth Annual Report of the Fibrodysplasia Ossificans Progressiva (FOP) Collaborative Research Project
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1996 was an extraordinarily exciting year in FOP research. A recent laboratory discovery shed the first new light on FOP in over 300 years. Investigators in the FOP laboratory identified the dramatic overexpression of bone morphogenetic protein-4 (BMP-4), a powerful bone forming hormone, in lymphoblastoid cells (a type of white blood cell or lymphocyte) of patients with the condition. This finding provided a tantalizing new connection between the blood-immune system and bone formation. Reports of this major discovery appeared in The New England Journal of Medicine and in Science in late August, 1996. A succinct summary of these findings appeared in the fall, 1996 issue of the FOP MILESTONES and is reproduced here in its entirety.

"NEW BREAKTHROUGHS IN FOP RESEARCH"

"The focus of FOP research continues to be on BMP-4, a powerful bone-inducing hormone. While this protein is vital for the formation of the human skeleton in FOP patients, it appears where one would not normally expect it. Heightened levels of the BMP-4 protein appear in the cell lines of active lesions of people with FOP. No other bone-inducing proteins appear to be present, nor does muscle tissue from those who do not have FOP reveal heightened levels of the protein.

While this is an extremely exciting discovery, originally this finding posed a puzzle for members of the University of Pennsylvania Working Group because even if lymphocytes (white blood cells) were releasing the protein, its presence in the body would be so diluted and short-lived that it could not explain the widespread bone growth seen in people with FOP. The biggest clue to the mystery came ironically from a biopsy sample taken 25 years ago. The sample revealed a large number of lymphocytes surrounding muscle cells. While this is a normal response to injury, the Working Group at the University of Pennsylvania theorized that this clumping could be what leads to the out-of-control bone growth seen in FOP. It is possible that the lymphocytes of people with FOP contain a genetic error that stimulates the production of BMP-4 and subsequent bone growth.

Now that the DNA code for the human BMP-4 gene has been found, studies are underway to determine what factors turn this gene on and off, and whether this gene alone can cause the complete clinical presentation associated with FOP. Researchers are also constructing a transgenic animal model to study the mechanism by which white blood cells may manufacture and deliver BMP-4 to muscle tissue. Elevated levels of a potent blood-vessel producing growth factor in the urine of FOP patients in the active phase of the disease support the hypothesis that blood vessel formation is a key to the formation of FOP swellings and subsequent bone formation. Researchers at the University of Pennsylvania are looking at possible new pharmaceutical agents which may inhibit this growth factor.

The recent discovery concerning overexpression of BMP-4 in people with FOP represents a major milestone for FOP research, and was recently published in the August 22, 1996 issue of THE NEW ENGLAND JOURNAL OF MEDICINE as well as the August 30, 1996 issue of SCIENCE. This exposure has enormous implications for FOP education of the medical community, exposing FOP to an extremely wide and diverse audience of physicians and scientists."

Based on these and other findings, laboratory studies have been extensive and have focused on four major areas:

- Genetic approach: Identification of the gene that is altered in FOP
- Molecular approach: Under standing the effects of over-expression of BMP-4 in FOP
- Physiology of bone formation
- Treatments for FOP

The remainder of the report will discuss the major projects and findings to date in each of these four areas.

GENETIC APPROACH

The genetic approach to understanding FOP is based on the finding that FOP is a genetic disorder. This means that FOP is caused by a gene that functions improperly. While we do not yet know if BMP-4 is the gene that harbors the mutation (genetic change) that causes FOP, we are certain that this gene is involved in the FOP process.

The goal of the genetic approach is to identify the gene that is mutated in persons who have FOP. The gene responsible for a genetic disease is usually identified by finding a known molecular (DNA) marker on the chromosomes that is co-inherited with the gene that causes the disorder. Such a marker will be located very near the gene of interest, providing an important localizing signal or "link". Such a localization procedure, known as linkage analysis, cannot be used in identifying the gene for FOP, since many large families with multiple affected members are needed to perform such an analysis, and such families do not exist in the FOP community. Therefore, an alternate approach must be pursued.

The alternate approach that we have undertaken (and that has successfully led to the identification of the BMP-4 gene in FOP) is called the candidate gene approach. This is essentially an educated guess approach as to what genes may be involved based on the clinical findings and natural history of the disease. Although definitive linkage analysis to confirm the involvement of a candidate gene cannot be performed in FOP, candidate genes can be excluded by the linkage process. In order to do that, DNA markers that are near the BMP-4 gene need to be identified and used to see if they follow the same exact inheritance pattern as FOP in the few small affected families that have been identified. If markers that are very close to the BMP-4 gene follow the same exact inheritance pattern as FOP in affected family members, that would provide support for BMP-4 as the causitive gene for the disorder. However, if linkage markers very close to the BMP-4 gene do not follow the same exact inheritance pattern as FOP in affected family mem-

bers, then the BMP-4 gene would be excluded from further consideration as the primary causative gene in FOP.

During the past year, we have found at least two important markers very near the BMP-4 gene that can be used in such an analysis. At present, we are determining whether or not the BMP-4 gene can be excluded as the genetically mutated gene in FOP. At the same time, we are also learning a great deal about other genes in the BMP-4 pathway. This same linkage exclusion process can be used to determine whether or not genes functionally related to BMP-4 could also be causative for FOP. If a gene is not excluded as a possible site of mutation by this approach, then the exact DNA sequences of the gene could be analyzed for the presence of a mutation. Eventually, an animal model containing the genetic mutation would have to be made in order to verify that the mutation could cause the disease.

Although the linkage analysis approach may not be as informative for FOP research as it has been for other genetic diseases which show inheritance through larger families, a genome-wide linkage analysis will nevertheless be useful to identify suspicious regions along the chromosomes that are of potential interest and importance for FOP. Through this approach, we may be able to use currently available genetic tools to identify suspicious regions and then use "molecular stealth technology" to pinpoint the specific gene of interest. The opportunity to perform this work is a direct offshoot of the Human Genome Project.

In summary, a novel combination of the candidate gene approach along with linkage exclusion analysis using new genetic linkage markers for the human BMP-4 gene, a genome-wide genetic linkage approach of candidate gene loci (including genes that are active in the BMP-4 pathway), and genetic sequencing of suspected mutations can prove to be extremely valuable in identifying the exact genetic mutation in the DNA that leads to FOP. Identification of the exact gene responsible for initiating the FOP cascade will open the door to develop targeted therapies and treatments for the disorder.

MOLECULAR APPROACH

The molecular approach to understanding FOP is based upon the finding that FOP is associated with overexpression of the BMP-4 gene. This information is critically important in understanding FOP and has been a direct result of the candidate gene approach described above. A major focus of work in the laboratory is based upon further elucidation of this finding. Our goal is to understand the exact circumstances, triggers, and effects of BMP-4 overexpression, and thereby identify cellular pathways that can be regulated therapeutically to impede the progression of the disease.

Increased levels of a gene product (RNA or protein) in a cell, indicate two possible causes: Either more of the gene product is made or usual amounts of the gene product are made but the product is more stable (less of the gene product is broken down over time). Work in our laboratory has verified that dramatic overproduction of BMP-4 RNA and protein in certain cells of patients who have FOP. The stability of the RNA and protein appears to be similar in affected and nonaffected individuals. This strongly suggests that the BMP-4 RNA and protein are the same in FOP as in individuals who do not have the condition, and we have in fact confirmed that to be the case in several affected individuals. These findings have directed our attention to the regulatory elements or "genetic switches" that turn on and turn off the BMP-4 gene. It is possible that one of these genetic switches is damaged in individuals who have FOP. It is also possible that one of the proteins that turns the switch on and off (which is made from a separate gene in a different part of the chromosomes) is abnormal. Understanding the on-off switches of the BMP-4 gene will have great value in designing treatments for FOP.

Our laboratory has identified several different forms of BMP-4 RNA (the BMP-4 intermediate product) in normal cells, which indicates that the on-off switches for BMP-4 also control which form of BMP-4 RNA product will be made. We are currently trying to understand the circumstances in which the body uses these alternate forms of BMP-4, perhaps at various times during development of the skeleton (in an embryo) or during bone repair (later in life).

It is quite possible that one of the alternate BMP-4 switches that is used during embryonic development fails to inactivate correctly and is reactivated later in life in people who have FOP. While we do not yet know if this is true, we are currently investigating this possibility intensively. Such a finding would help us to design dramatically better medications to treat FOP by turning off the abnormal switch that may be either "trigger sensitive" or "stuck in the on position."

Intensive study is also underway to identify the receptor or "lock" that accepts the BMP-4 "key" in connective tissue cells of patients who have FOP and in unaffected individuals. In order to understand the effects of alternate forms of BMP-4 on signaling, it is essential to understand which cells have the BMP-4 receptor to receive the message (in other words which cells have the lock that fits the BMP-4 key). Such knowledge would also be critical in the development of effective therapy.

Although the tools are available to distinguish between the BMP-4 and BMP-2 genes and their intermediate RNA products, the BMP-4 and BMP-2 proteins are remarkably similar. Antibodies are the tools that are used to specifically identify these important protein products, and additional work in the laboratory is focused on developing special antibodies to detect human BMP-4 and distinguish it from human BMP-2.

PHYSIOLOGY OF BONE FORMATION

Currently there are six major animal models that we are using to understand the process of bone formation in FOP. First, we are using recombinant (genetically engineered) BMP-2 and recombinant BMP-4 protein to stimulate bone formation and to study the earliest events of bone formation (those that occur within a matter of minutes and hours after implantation)! It is essential to understand these extremely early events in order to better control them. One of the extremely important findings from our work so far is that new blood vessels are formed at a very early stage in BMP-induced bone formation. These primitive blood vessels are seen in the early FOP fibroproliferative lesions (the lumps and bumps and tumors that appear on the neck and back early in life) and strongly suggests that BMP induces these blood vessels (either directly or indirectly). The formation of blood vessels is essential for the formation of bone, and therefore, if blood vessel formation could be blocked, it might be possible to prevent ectopic bone formation in FOP.

In a related study, a powerful blood vessel forming hormone has been found in the urine of children and adults

who have FOP at times of acute flare-ups of the disease (but not when the disease is quiescent). This potent hormone is called basic fibroblast growth factor. Basic fibroblast growth factor (bFGF) and BMP-4, are not only potent co-stimulants of bone formation, but are also extremely potent inhibitors of muscle cell differentiation. Therefore these two powerful signals may work together in a type of "molecular partnership" to inhibit the development of muscle and stimulate the development of bone during the normal formation of the skeleton. But, they may also work together in a type of "molecular conspiracy" later in life to inhibit muscle repair and stimulate ectopic bone formation in FOP. This work is being pursued in collaboration with investigators at Harvard University.

In another model, we have developed animals transgenic for BMP-4 by over-expressing the BMP-4 gene in circulating lymphocytes (a type of blood cell). Currently, we are over-producing BMP-4 in the B lymphocytes and will follow these studies by over production of BMP-4 in the T lymphocytes. Then, we will breed these transgenic animals to examine if over production of BMP-4 can be sustained in all of the lymphocytes. (Both B and T lymphocytes have been identified in the early FOP lesions.) If lymphocytes mediate osteogenesis by over-producing and secreting BMP-4, then it is possible that both types of lymphocytes will be necessary to see the effect.

An FOP-like condition has been identified in the cat and also shows evidence of BMP-4 over production by lymphocytes in the early lesions. Unfortunately, no live cats with the FOP-like condition are available so our current studies must be done on fixed and preserved tissues. Articles have been published recently in the veterinary literature to alert clinicians to the existence of such cats. This work is being pursued in collaboration with investigators at Cornell University.

A fourth important animal model in our study of FOP involves the examination of the powerful protein called Fos, an important regulator of the BMP-4 gene. Early work in our laboratory has identified the over-production of Fos and BMP-4 in animals that have an FOP like condition, but only the over expression of BMP-4 in humans who have FOP. The role that Fos and other related molecules play in regulating the BMP-4 gene are extremely important to understand in trying to decipher the mechanisms by which BMP-4 over-expression may lead to ectopic bone formation. This work is being pursued in collaboration with investigators at the University of London in England, and the Institute for Molecular Pathology in Vienna, Austria.

The fifth animal model under development involves the implantation of bone marrow stem cells from patients who have FOP into mice that are genetically engineered to accept such a stem cell transplantation. This model will help test the hypothesis that bone marrow cells in patients who have FOP give rise to cells that circulate in the blood, escape into muscle and over-produce BMP-4 leading to ectopic bone formation. Since it is unsafe to obtain bone marrow cells from FOP patients by direct needle aspiration, the bone marrow cells will be coaxed out of the bone marrow by the use of a special medication (called granulocyte colony stimulating factor) and collected from the peripheral blood of FOP patients. These cells will then be injected into mice that are engineered to accept these cells. The mice will be studied over time to see if they develop an FOP-like condition.

Finally, important clues have been forthcoming over the past year from studies by others on the expression of the BMP-4 gene in chickens and ducks. BMP-4 is essential in forming the separations between the digits in a chicken foot and in a human hand. Amazingly, the foot of a chicken can be turned into a webbed duck-like foot by blocking the action of the BMP-4 gene. Even more amazingly, the BMP-4 protein is missing from the webbed foot of a duck! Thus, it appears that BMP-4 (in addition to regulating bone formation) also regulated programmed cell death (apoptosis) that allows separate, non-webbed digits to form in the chicken limb as in the human hand (but not in the duck foot)! This finding provided a very important clue in understanding FOP. It is well known (and has been observed by many families of children who have FOP) that the early lumps and bumps, especially those that form on the neck and back, may form within hours and become extremely large; but they may also disappear as rapidly! How could such a large FOP lesion disappear so quickly? It is likely that programmed cell death (apoptosis) plays a role in the rapid disappearance of the FOP lesions. We have performed preliminary studies to see whether programmed cell death plays a role in the regression of fibroproliferative lesions, and indeed it does!!! Thus, BMP-4 appears to play a major role not only in the formation but also in the regression of the early FOP lesions!! This raises a critical question: what factors determine whether BMP-4 leads to the formation of a new piece of bone or whether it leads to the regression of a pre-bone lesion? And furthermore, could we use that knowledge to direct the molecular processes to regress a lesion rather than to form an extra bone? Thus, it is possible, that we may be literally standing on the answer to FOP by understanding how BMP paradoxically regulates both the formation of bone and the programmed cell death (apoptosis) that leads to the regression of the pre-bone lesions and the interdigital web space, and possibly the toe malformations! In the coming year we will focus attention on BMP-4 induced apoptosis and the molecular regulation of tumor regression in the early FOP lesions.

TREATMENTS FOR FOP

The ultimate goal of all FOP research is to apply knowledge from the laboratory to the treatment and cure of FOP. Two novel forms of therapy are currently under study in the laboratory. Each one is based upon molecular clues that have been discovered in the FOP laboratory and each one will require detailed animal and human toxicity studies before they are applicable to the potential treatment of FOP.

The first therapeutic agent called squalamine, is a unique amino-sterol from the shark. Squalamine is a very powerful drug in stimulating the regression of newly formed blood vessels. Since blood vessels are essential for the formation of bone, and are seen in early BMP-induced lesions (as well as in early FOP lesions), it is plausible that squalamine may have an effect on inhibiting one of the earliest events of bone formation. Early experiments with squalamine have shown a dose-response effect in stimulating the regression of early blood vessels related to bone formation. During the next year, intensive studies will be performed to determine the exact dose and timing necessary to stimulate the regression of new blood vessels associated with BMP-induced bone formation. Other preliminary investigations with squalamine indicate that it is an extremely potent agent in stimulating the regression of blood vessels associated with certain

brain tumors and therefore its potential use in humans will be propelled by this finding as well. Toxicity studies using squalamine in humans will be underway within the next year and may help pave the way to the use of squalamine for the treatment of FOP.

Last year, scientists from the University of California at Berkeley who study the earliest embryonic development of the central nervous system (the brain and the spinal cord) discovered that this important process was dependent on the total exclusion of BMP-4 from that area of the developing embryo. The scientists discovered that a protein produced in the primitive brain region of the developing embryo was responsible for binding and inactivating BMP-4 and thus prohibiting it from reaching the cells that will become the central nervous system. Two proteins, called chordin and noggin, that seem to share this property of avidly binding BMP-4 have now been identified. The underproduction of noggin or chordin at specific times during embryonic or post-natal development leads to extra BMP-4, and to ectopic bone formation. These findings pave the way for testing the use of chordin and/or noggin in binding and inactivating extra BMP-4 in conditions like FOP. Collaborators are beginning studies to examine the effects of noggin on inhibiting BMP-induced bone formation in animals. Based upon the results of those studies, noggin might eventually be developed as a therapeutic agent in the treatment of BMP-4 induced bone formation in FOP. Thus, a direct finding from the laboratory combined with important findings from other research groups around the world have led to new collaborative efforts aimed at combating the ectopic bone formation in FOP.

Jules Rosenstirn, a physician from San Francisco, wrote about FOP in 1918: "one does not wonder that a disease so baffling in its course from the first causes to its ultimate state, should invite the speculative as well as the patiently investigating observer to lift the obscuring veil and solve this embarrassing puzzle." While we still have far to go, it is quite evident that recent discoveries from the FOP collaborative research project have provided a much firmer basis for understanding the molecular basis of BMP-induced bone formation, and have thus enabled us to consider more appropriate molecular means for establishing effective treatments, preventions, and cures for FOP.

CAUSE AND CURE

Cause and cure are the two words that propel the international FOP collaborative research effort and provide the guiding principle for all we do: to discover the exact molecular cause of FOP and to use that knowledge to develop therapies that will be truly effective in preventing, treating and curing the disease. 1996 was a year of spectacular progress and promise. We hope for even more progress in 1997.

As we have said before, "finding the cure for FOP is not a job, it is a mission." We are all extremely proud to be part of that mission and enormously grateful to those who support this vital research effort:

The International FOP Association
The Orthopaedic Research and Education Foundation
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The Ian Cali Fund for FOP Research
The Isaac and Rose Nassau Professorship of Orthopaedic Molecular Medicine
The many individuals, families, and friends throughout the world who contribute to the FOP effort.

We will continue to need your help until a cure is found. Thank you.