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13. ABSTRACT (Maximum 200) <p style="margin-left: 40px;">The long term goal of this research program is to develop fibroblast growth factor-1 through rational protein engineering into a potent and specific anabolic agent for the treatment of osteoporosis and fracture repair. The specific aims of this research plan are:</p> <ol style="list-style-type: none"> 1) To evaluate the effects of existing mutant forms of FGF-1 on bone cells <i>in vitro</i>, on bone formation <i>in vivo</i> and to assess their toxicological or undesirable effects. 2) To generate additional FGF-1 mutants or chimeric proteins that are likely to exhibit enhanced anabolic activity on bone with reduced toxicological effects. During the current year of support we have made significant progress with regard to these specific aims. The data obtained to date indicate that the proposed research plan was realistic and rational. We have generated five different FGF-1 variants and shown utility of some of these variants <i>in vivo</i>. The results of the <i>in vivo</i> and <i>in vitro</i> studies indicate a logical progression towards optimizing the anabolic activity of the protein for bone will be possible. The cys-free mutant of FGF-1 has been studied extensively and this construct is likely to become the parent protein to which other mutations will be incorporated. 			
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FOREWORD

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Wilson H. Berger 10/6/97
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INTRODUCTION

The long term goal of this research program is to develop fibroblast growth factor-1 via protein engineering into a potent and specific anabolic agent for the treatment of osteoporosis and fracture repair. Osteoporosis is a disease which afflicts nearly 200 million people worldwide and this number is expected to double in the next 25 to 35 years. It is probable that all people with the disease will ultimately benefit from treatments to increase bone formation. A more acute and relevant need for an anabolic treatment is a prophylactic for the approximate 700,000 hip fractures each year in the United States, Europe and Japan combined. The greatest therapeutic challenge in the osteoporosis field at the present time is identification of an agent that promotes significant bone formation. Although there are effective resorption inhibitors for osteoporosis (bisphosphonates, estrogen and calcitonin), these drugs essentially stabilize bone mass and do not cause substantial increases in bone mass or restore trabecular bone microarchitecture. For patients with severe and established osteoporosis, there is thus a need for therapeutic agents which stimulate bone formation and initiate the cascade of events involved in osteoblast differentiation. Those agents which are currently known to have a stimulatory effect on new bone formation are fluoride, low-dose intermittent parathyroid hormone and its analogs, and the peptide growth factors which are incorporated into the bone matrix and released from bone as it resorbs.

During the past several years, it has become apparent that members of the fibroblast growth factor (FGF) family of ligands and receptors are essential for normal skeletal growth (Rousseau, et al., 1994). Recent observations demonstrate that a variety of inherited disorders of skeletal growth are due to point mutations in FGF receptors. It is also known that members of the FGF family are expressed by bone cells, stored in the bone matrix and stimulate bone formation *in vivo*. Our preliminary data indicate a significant osteogenic potential of systemic administration of FGF-1 in animal models of osteoporosis. There are also certain toxicological or undesirable affects associated with these treatments. The results obtained to date indicate that the therapeutic window is relatively narrow. We have generated a variety of mutant forms of FGF-1 that appear to be better candidates for the proposed therapeutic uses. We believe that a further evaluation of existing mutants and production of additional mutants or chimeric proteins will improve the efficacy of the FGF-1 derivatives as anabolic factors in the treatment of osteoporosis and other disorders of bone metabolism.

The original specific aims of the proposal were:

- 1) To evaluate the effects of existing mutant forms of FGF-1 on bone cells *in vitro*, on bone formation *in vivo* and to assess their toxicological or undesirable effects.
- 2) To generate additional FGF-1 mutants or chimeric proteins that are likely to exhibit enhanced anabolic activity on bone with reduced toxicological effects.

Progress towards these aims achieved during the first year of funding are summarized below.

BODY

Based on the original "statement of work" progress during the first year was excellent. The animal models and histomorphometric assays have been established at the Holland Laboratory, large scale production of specific FGF-1 mutants and chimeric proteins have been completed and tested *in vitro* and *in vivo*. Based on these data, second generation molecules have been identified that will be tested in the coming year. A summary of these results is provided below.

A major problem in the large scale production of several of the FGF-1 mutants in prokaryotes was the fact that the majority (>95%) of the recombinant protein ended up in the inclusion bodies of the bacteria. The protein could be solubilized with urea, but could not be purified in an active state following simple dialysis. We found that the mutant FGF-1s maintained a relatively high affinity for immobilized heparin even in the presence of 6M urea. The solubilized proteins were adsorbed to heparin-Sepharose in the presence of urea, then a reverse gradient of 6M and 0M urea buffers were passed through the column. Biologically active protein could then be eluted with a normal NaCl gradient. We infer that the bound heparin allows the protein to refold in an active conformation as the urea is slowly removed during the first gradient step. We can now produce 50-100 mg quantities of all the recombinant proteins we have constructed from a single prep.

The mutant or chimeric forms of FGF-1 produced to date are:

- 1) cys-free - each cysteine residue (3) changed to serine.
- 2) S330K - the serine at position 130 changed to a lysine residue.
- 3) S31K - the serine at position 31 changed to a lysine residue.
- 4) HBGAM chimera - a fusion of the protein HBGAM (also known as bone specific protein 1) to the amino terminus of FGF-1.
- 5) HAFGF-1 - the 9 residue hemagglutinin epitope fused to the carboxy terminus of FGF-1.

The rationale and the status of each of these constructs are detailed below:

1) cys-free: studies of the cys-free mutant were a major focus of the original application and represents the FGF-1 variant that is farthest along at this point. It is likely that the future mutants will utilize the cys-free construct as the parent factor. We have completed 1 *in vivo* study comparing the cys-free mutant to wild-type FGF-1 in the ovariectomized (OVX) rat model of osteoporosis. The preliminary data on systemic FGF-1 in this model in the original application involved daily treatment for 28 days. In the present study we used a more realistic regimen of 28 week-day injections over a total of 38 days. The study utilized 200 or 20 $\mu\text{g}/\text{kg}$ of wild-type and cys-free FGF-1. The injections were started two weeks after ovariectomy. At the conclusion of the study the rats were euthanized and femurs, tibia, vertebrae, spleen, kidney, liver and thymus retrieved for study. Histological analysis of the vertebrae and tibia by H&E staining of sections of demineralized tissue revealed a dramatic loss of bone mass in the untreated animals. The bone mass of both 200 $\mu\text{g}/\text{kg}$ groups was normal. There was a noticeable loss of bone mass in the 20 $\mu\text{g}/\text{kg}$ wild-type treated animals that was not apparent in the cys-free group. These

results appeared consistent with the *in vitro* and local *in vivo* studies presented in the original proposal. We also examined the strength of the femurs of these animals in a 3 point bending assay. It should be noted that such functional assays were not a part of the original application. We perceived this to be a weakness of the project. Though a collaboration with a mechanical engineering Ph.D. student from the University of Maryland, Baltimore County, we were able to establish this study. The results are summarized in Figure 1 (appendix). The data represents the force in pounds required to break the femurs of animals from the different treatment groups. It can be seen that the OVX animals exhibit ~75% reduction in bone strength when compared to normal rats. It is also clear that the 200 or 20 $\mu\text{g}/\text{kg}$ treatments of both wild-type and cys-free FGF-1 were able to maintain (or establish) normal bone density in the OVX animals. This result was not anticipated based on the histological analysis, but presents a pleasant surprise. An identical study is presently in progress using animals that were aged for three months following ovariectomy. This study will provide a clear analysis of the relative efficacies of the two doses of the two proteins to restore bone mass and density.

2) S130K: Studies of this mutant have not yet been conducted *in vivo*. *In vitro*, this mutant is more specific for target cells that correlate with *in vivo* performance. Our strategy in the next year is to incorporate this mutation into the cys-free construct.

3) S31K: Studies of this mutant are also limited to *in vitro* assays. We have a manuscript in preparation (will be sent at time of submission) that demonstrates increased affinity of this protein for immobilized heparin with no change in mitogenic activity. We believe that increased heparin affinity will improved the targeting of the protein to bone in local and perhaps systemic administration. This hypothesis remains to be tested.

4) HBGAM chimera: Studies of this construct have included *in vitro* and local *in vivo* experiments. The rationale for the construction of this chimeric protein was based on the reports that HBGAM is found at relatively high concentrations in bone (Gieffers, et al. 1993) and that osteoblasts have a relatively high number of cell surface receptors for the protein. Our *in vitro* data demonstrates that the chimeric protein is relatively independent of exogenous heparin for maximal mitogenic activity when compared to wild-type FGF-1 (Hampton, et al. manuscript in preparation). Local injections of the chimera over the calvaria of mice result in new bone formation similar to that seen with FGF-1. A major goal of the next year of support is to determine the effectiveness of this construct in systemic studies and to determine whether the chimera in conjunction with the cys-free and other mutants should become the base or parent construct for future studies.

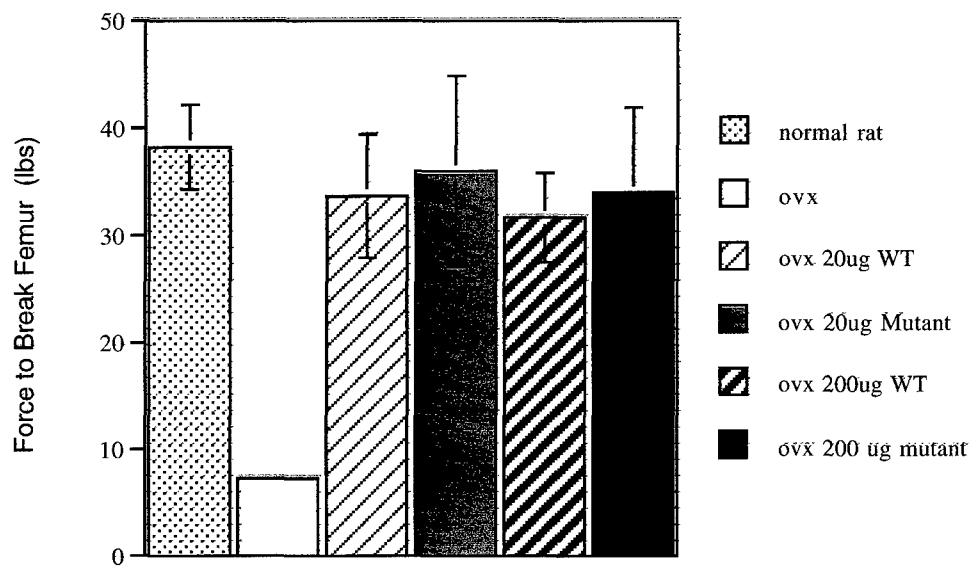
5) HAFGF-1: This construct was produced to take advantage of the high quality antibody that we have generated to the HA epitope. The antibody will have utility in quantitating the localization of HAFGF-1 to bone and in studies of the distribution of the protein following systemic administration. The use of this construct and the HA antibody for these studies will allow us to discriminate between endogenous and administered FGF-1.

CONCLUSIONS

In summary, progress on the specific aims has been good. The addition of the 3 point bending assays to the methods of approach is important. The data generated with the cys-free mutant indicates that it should become the parent compound for future mutations. We believe the data obtained to date supports our hypothesis that systemic FGF-1 can become an effective therapeutic agent for this treatment and prevention of osteoporosis. We look forward to continued support and continued progress in this critical area of research.

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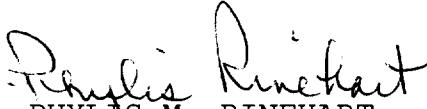
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