Alteration of the Connective Tissue Network of Striated Muscle in Copper Deficient Rats

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T. K. BORG, L. M. KLEVAY, R. E. GAY, R. SIEGEL AND M. E. BERGIN. Alteration of the Connective Tissue Network of Striated Muscle in Copper Deficient Rats. Journal of Molecular and Cellular Cardiology (1985) 17, 1173–1183. The connective tissue network in striated muscle, consisting principally of collagen is arranged in a three dimensional network and is intimately associated with muscle function. Previous studies have shown that animals maintained on a copper-deficient diet undergo myocardial hypertrophy and exhibit cardiovascular lesions such as ventricular aneurysms that eventually rupture. A deficiency of copper in the diet is known to inhibit lysyl oxidase, a metalloenzyme requiring copper as a cofactor and which is also responsible for collagen and elastin crosslinking. Examination by scanning and transmission electron microscopy of skeletal and cardiac muscle from rats maintained on copper-deficient diets showed both gross and microscopic lesions to the connective tissue network. Immunohistochemical staining by light microscopy with antibodies against lysyl oxidase showed that the enzyme was equally present in both control and experimental animals. Fluorescent staining for antibodies against collagen types I and III showed similar results. From these studies we concluded that the collagen secreted during hypertrophy was not crosslinked by lysyl oxidase due to the absence of the copper cofactor. This resulted in the failure of the connective tissue network to transmit and distribute the increased force associated with myocardial hypertrophy and resulted in myocardial aneurysms.

KEY WORDS: Collagen; Copper deficiency; Skeletal muscle; Hypertrophy of the heart; Lysyl Oxidase.

Introduction
The connective tissue found in striated muscle, which consists primarily of collagen, is arranged in a precise, three dimensional network [4–8, 44, 53]. This network is composed of a perimysium of large wavy bundles of collagen which connect to the endomysial collagen that surrounds the myocytes. The endomysium in turn can be divided into at least four components: (1) a dense weave network that surrounds myocytes; (2) collagen struts 120 to 150 nm in diameter that connect adjacent myocytes; (3) similar size struts that connect myocytes and capillaries; and (4) individual collagen fibrils, microthreads, glycosaminoglycans, and glycoproteins of the extracellular matrix [6, 8, 10, 44]. These components of the connective tissue network are intimately associated with muscle function. The myocyte-myoctye and myocyte-capillary struts aid in myocyte alignment and capillary patency during the contraction cycle [6, 10]. The endomysium weave and the perimysium function together, along with the other components, to form an elastic stress tolerant network [6, 8, 10, 52]. The purpose of this network is to distribute the force generated by muscular contraction [6, 8, 10, 53].

Although anemia has been associated with copper deficiency since the establishment of
copper as an essential nutrient [20], sometimes anemia is not severe [1, 12, 23, 25]. Recent research has been directed toward the metabolism of lipids [1, 2, 21, 37], uric acid [29], glucose [11, 22, 30], and abnormalities of the electrocardiogram [31, 49]. Cardiovascular damage in deficiency can be extensive and has been recently reviewed [49]. The gross cardiovascular lesions in several species maintained on copper-deficient diets included myocardial hypertrophy, focal necrosis, aortic rupture, fibrosis, ventricular aneurysms, and eventual rupture of ventricular myocardium [1, 19, 26, 38-40, 46, 49, 50, 51]. A deficiency of copper has been shown to inhibit the enzyme, lysyl oxidase, a metalloenzyme that requires copper as a cofactor and is responsible for collagen and elastin crosslinking [18, 24, 42, 47]. Recent data have shown there was an alteration in the ratio of type I to type III collagen produced in copper-deficient rats [13]. Although the total collagen was similar between treated and control animals, differences in the pepsin extractable collagen was greater in the copper deficient animals [13]. Copper is also required for hematopoiesis as well as other metabolic effects [1, 2, 11, 20-22, 29, 30, 37].

Alteration of the connective tissue network in neonatal rats treated with the lathyrinic agent, β-aminopropionitrile (BAPN), which inhibited lysyl oxidase, resulted in lesions in the heart similar to those reported to occur in animals maintained on copper-deficient diets [1, 4, 17, 45, 46]. The connective tissue network failed to form as the heart underwent hypertrophy during neonatal growth, the connective tissue network was not able to distribute the increased force of contraction to the ventricular wall, and the myocardium eventually ruptured [7]. The purpose of this study was to determine if the lesions in the myocardium and skeletal muscle in adult rats maintained on a copper-deficient diet were related to the alterations of the connective tissue network.

Materials and Methods

Experimental data
Two experiments were done with male weanling rats of the Sprague-Dawley strain obtained from Sprague-Dawley, Madison, WI. In each experiment 20 rats were matched by weight into two equal groups. The mean weight for the first experiment was 51 g and the mean weight for the second experiment was 62 g. Mean differences in weight between groups were equal to or less than 0.5 g; mean absolute differences in weight between pairs of rats were equal to or less than 0.7 g. The environment was similar to that described by Klevay et al. [32].

The purified diet [27] used in these experiments has been in use for over a decade. It is based on sucrose (62% by weight), egg white (20%), and corn oil (10%); and it contains no cholesterol. The diet contains all the nutrients known to be essential for rats, including 2 mg of biotin/kg [28], but without supplementation it is deficient in both copper and zinc.

Diets were supplemented with drinking solutions [27] of reagent grade CuSO₄·5H₂O and Zn(C₂H₃O₂)·2H₂O in distilled demineralized water. Drinking solutions contained either 10 μg zinc and 2 μg Cu/ml (supplemented) or 10 μg Zn/ml (deficient). Cholesterol in plasma was measured by fluorometry according to Carpenter et al. [9]. Means were compared with the Student's t-test [47]. After hypercholesterolemia was detected, the rats were shipped from Grand Forks, N.D. to Columbia, S.C. Animals were killed within 2 days of arrival.

Microscopy
Animals were anesthetized after 30 to 40 days on the diets, the hearts rapidly removed, and prepared for light and electron microscopy. Skeletal muscle [soleus, plantaris, and extensor digitorum longus (EDL)] were also excised and prepared for light and electron microscopy at this time. These procedures have been well documented (4 to 8). Briefly, the heart was cut into 1 to 2 mm slices perpendicular to the midline, in a 4% phosphate buffered (0.1 M, pH 7.4) glutaraldehyde. Specimens were rinsed in buffer, post-fixed in 2% OsO₄, dehydrated in a graded series of acetone and either embedded in Epon for transmission electron microscopy (TEM) or critical point dried for scanning electron microscopy (SEM). Scanning electron
microscopy samples were sputter coated with gold and examined in a JEOL JSM-35 SEM. Samples for TEM were thick sections (1 μm) and stained with 1% toluidine blue for light microscopy, or thin sectioned, stained with uranyl acetate, and examined on a JEOL 100B transmission electron microscope.

**Immunohistochemistry**

Rat heart and skeletal muscle were removed, rapidly frozen in liquid nitrogen, warmed to −10°C and frozen sections cut at 4 to 6 μm for immunohistochemical staining. Sections were stained using rabbit antisera against collagen type I and III as described by Gay et al. [15, 16] and Borg et al. [10]. Monoclonal antibodies against lysyl oxidase were produced and assayed according to the procedures of Siegel [48]. Fluorescent staining was carried out by the indirect method using fluorescein isothiocyanate (FITC) conjugated to anti-rabbit IgG or FITC goat anti-mouse for monoclonal antibodies. Sections reacted with pre-immune rabbit sera were used as controls.

**Results**

After 30 to 41 days on the copper-deficient diet, hypercholesterolemia and anemia (Table 1) were found. Several of the animals in the experimental group had ventricular aneurysms; some of these ruptured to produce hemothorax, whereas no control animals exhibited these features.

**Skeletal muscle**
The skeletal muscle grossly appears to be atrophied in deficient animals but not in the (supplemented) controls. Differences in the amount of connective tissue, but not in the basic arrangement of the connective tissue, were observed among the three types of skeletal muscles (Figs 1 to 4). The control soleus muscle, which is a slow twitch muscle, has the most well-developed connective tissue network of the three skeletal muscles observed (Fig. 1). Large bundles of collagen forming the perimysium connected to a dense weave network (endomysium) that surrounded the myocytes. Other components of the endomysium, myocyte-myocyte and myocyte-capillary struts, were also well developed in the control soleus muscle. The fast twitch muscles, EDL and plantaris, showed less well-developed connective tissue networks (data not shown).

The connective tissue of skeletal muscles from deficient animals was poorly developed (Fig. 2). The local association of the perimysial collagen with the endomysial collagen was the most striking feature of skeletal muscle from copper-deficient animals compared to the tight association seen in supplemented animals. The endomysial weave collagen was not well developed in deficient animals (Fig. 2). No specific defect in the connective tissue network could be localized to any particular component. The lack of development of the endomysium was most apparent in the soleus because of the amount of collagen present in this particular type of muscle.

**Heart**

Low magnification SEM and light microscopy showed distinct separation of the layers of myocytes in the copper-deficient animals (Fig. 4). Examination of the heart at higher magnification indicated similar phenomena seen in the different types of skeletal muscles. The endomysium, compared to control (Fig.

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**TABLE 1. Cholesterol in plasma (mg/dl) and hematocrits (%)**

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<thead>
<tr>
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<th>Experiment 1</th>
<th>Experiment 2</th>
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<tr>
<td></td>
<td>Cholesterol</td>
<td>Hematocrit</td>
</tr>
<tr>
<td>Deficient</td>
<td>135 ± 6.2</td>
<td>29 ± 0.9</td>
</tr>
<tr>
<td>Supplemented</td>
<td>110 ± 3.8</td>
<td>44 ± 0.6</td>
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<tr>
<td>t</td>
<td>3.39</td>
<td>14.3</td>
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<tr>
<td>P</td>
<td>&lt;0.01</td>
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*Mean ± s.e., ten animals per group.
FIGURE 1. Scanning electron microscopy (SEM) of the soleus from a control rat showing the bundles of myocytes (M) surrounded by the perimysial and endomysial collagen (C; arrows). × 400.

FIGURE 2. Scanning electron microscopy of the soleus from a copper deficient rat. The collagen (C; arrows) does not interconnect the myocytes (M) as indicated by the tangled appearance. Compare with Figure 1. × 400.
FIGURE 3. Scanning electron microscopy of the left ventricle of rat maintained on a control diet showing the myocytes (M) and perimysial collagen (P). Myocytes are parallel with no separations at the lateral margin. × 450.

FIGURE 4. Scanning electron microscopy (SEM) of the left ventricle of rat maintained on a copper deficient diet showing the myocytes (M) and the absence of perimysial collagen. Note the separation of myocytes along their lateral margins (arrows). Compare with Figure 3. × 500.
FIGURE 5. Transmission electron microscopy (TEM) of the left ventricle of a copper deficient rat showing normal myocyte ultrastructure both intracellularly. Intercellular bundles of striated collagen fibrils are evident in the extracellular matrix (arrows). × 15,000.

FIGURE 6. Transmission electron microscopy (TEM) of the left ventricle of a rat maintained on a copper deficient diet showing the distorted contractile elements in the myocyte (M). Swollen mitochondria, and large intracellular spaces. The extracellular matrix contains electron dense, flocculent material as well as a few bundles of banded collagen (arrow). × 15,000.
Connective Tissue of Copper Deficient Animals

3) was poorly developed and large spaces between individual myocytes were apparent (Fig. 4). Large areas lacking in endomysial weave and myocyte-myocyte struts were observed in some regions. The incomplete development of the connective tissue network was not uniform throughout the myocardium. Areas of the septum of copper-deficient animals were similar to control animals, whereas areas of the free wall contained a poorly developed connective tissue network. Some regions of skeletal and myocardium showed abnormal appearing myocytes and distorted connective tissue (Figs. 2, 4, 6).

Examination of the myocardium of animals maintained on copper-deficient diets by light and TEM indicated that there were regions of ischemia and necrosis (Fig. 6). Large, misshapen mitochondria, nonaligned Z-bands, and separation of the contractile fibers at the Z-band were apparent (Fig. 6). The separation of myocytes seen by SEM was also apparent showing increased areas of extracellular matrix. Transmission electron microscopy of the copper-deficient myocardium showed numerous unbounded microfibrils, single collagen fibrils, a few bundles of loosely associated bundles of banded collagen fibrils and large amounts of electron dense material which coated the collagen fibrils as well as occupied the extracellular space. The collagen bands tended not to be uniform as large areas of the fibrils contained amorphous regions (Fig. 6). There appeared to be more collagen in the extracellular matrix of supplemented animals than in the deficient animals. Transmission electron microscopy of control animals usually showed only bundles of tightly associated collagen fibrils and occasionally single collagen fibrils (Fig. 5). Banding of collagen in control animals was always uniform. The electron dense material observed in the extracellular matrix of supplemented animals was not as abundant as in the copper-deficient animals and was associated with the outer fibrils of collagen in bundles (Fig. 5).

Immunohistochemistry

Immunohistochemistry staining for lysyl oxidase and collagen types I and III in both skeletal and cardiac muscle showed that there were no differences between copper-deficient and control animals (Figs 7 and 8). Although slight differences in the staining pattern were observed in the copper-deficient sections, the fluorescent staining for types I and III collagen showed that both antigens were present in the heart and skeletal muscle (data not shown). No differences in the intensity or

FIGURE 7. Immunofluorescent pattern of anti-lysyl oxidase from cardiac muscle of a rat maintained on the copper deficient diet. The fluorescence is confined to the extracellular matrix. Similar patterns were observed in cardiac muscle. x 600.
pattern of staining were observed between copper-deficient and controls when stained with monoclonal antibodies against lysyl oxidase. The staining pattern for all antigens was always confined to the extracellular space.

**Discussion**

Copper deficiency in these rats was verified by the finding of hypercholesterolemia, anemia, hemotherax and aneurysm. In experiment two, the hypercholesterolemia did not reach statistical significance ($P > 0.05$). The animals were shipped for anatomical study because the finding of a rat with hemotherax in Grand Forks made a delay in the hope of increasing the lipid abnormality undesirable. Rats at this stage of deficiency die suddenly [1, 31, 49], often with hemotherax, aneurysm and ruptured hearts [49]. Rats in similar experiments have decreased copper in heart [36] and skeletal muscle [35].

The lesions of cardiac and skeletal muscle in the deficient animals probably were produced by several mechanisms. Anemia can produce tissue anoxia which often produces increased cardiac output [14]. In contrast, hearts deficient in copper develop less systolic pressure and beat more slowly *in vitro* [45] than supplemented hearts. This impairment may be the result of anoxia, or depletion of norepinephrine [43], adenosine triphosphate [33] and cytochrome oxidase [17, 23]. These latter characteristics may contribute to the focal myocardial necrosis [1, 46]. Collectively, these defects may make the copper deficient heart less able than normal to withstand systolic pressure.

Perhaps the most important harmful effect of myocardial copper deficiency is an impairment of collagen crosslinking because of too little copper for lysyl oxidase (*vide supra*), the only copper-requiring enzyme known to be necessary for collagen fibrillogenesis [42, 47]. The lack of collagen crosslinking would alter the strength of the collagen network and would not allow the connective tissue network, in the form of the endomysial weave network, to distribute the force of contraction to the ventricular wall. Rupture of a free ventricular wall composed of hypertrophied myocytes associated with fibrils of insufficiently crosslinked collagen seems likely and has been reported [1, 37].

Dawson et al. [13] found that net collagen synthesis in hearts of weanling rats during 12 weeks of copper depletion was unimpaired; however, crosslinking of type III collagen was impaired more than that of type I. This differ-
ential impairment may explain the presence of apparently normal perimysial collagen bundles. The perimysial collagen is thought to be primarily composed of type I, which is less elastic than type III, the primary collagen of the endomysium [7]. Developmental studies in the heart indicate that the type I collagen of the perimysium is present early in the postnatal development [7]. These observations have been confirmed in similar studies using peptic digestion of the collagens [13]. Increased amounts of collagen in the endomysium have been reported in hearts undergoing hypertrophy.

No morphometric data are available to indicate which regions of the heart are undergoing hypertrophy. If hypertrophy is asymmetrical, it would account for the focal necrosis and the patchy appearance of normal and abnormal regions of the connective tissue. The cellular debris observed in SEM examination of copper-deficient muscle may have been due to the rupture of these areas of focal necrosis which are present in copper-deficient muscle [7]. The free wall of the left ventricle showed more abnormal arrangement of the connective tissue network than did the septal region, indicating that there may have been a regional response of hypertrophy as has been described in some types of hypertrophy [7].

The atrophy of the skeletal muscle observed in copper-deficient rats is probably similar to disuse atrophy. Some of the metabolic deficits enumerated for cardiac muscle also may occur in skeletal muscle. The animals appeared lethargic in the cages, not displaying normal movement behavior in the cages. Differences in the amount of connective tissue between control fast twitch (ELD and plantaris) and slow twitch (soleus) muscle are probably due to the physiological functions of the muscle [37]. With atrophy, the connective tissue network does not appear to decrease in volume as the myocytes do. This would result in greater stress placed on the connective tissue network and may result in breakage under conditions which cause atrophy, accounting for the loose appearing connective tissue network and the lack of myocyte alignment.

From the fluorescent immunohistochemical staining, types I and III collagen and lysyl oxidase were present in copper-deficient and control animals. In the case of lysyl oxidase, the enzyme was present but not active in crosslinking collagens. These results indicate the morphological presence of the antigens but biochemical studies are necessary to define the status of these macromolecules. The degree of crosslinking and the rate of collagen synthesis need to be investigated, as well as the glycosaminoglycan content of copper-deficient and control muscle.

Results observed in this study were similar to those observed in animals treated with the lathyritic agent, BAPN [37, 50]. The mode of action of BAPN is to also inhibit lysyl oxidase but apparently in a different manner than does copper deficiency [50]. Our observations indicate that additional biochemical and immunohistochemical experiments are necessary to further examine the effect of dietary deficiency on the structure and function of the connective tissue network in cardiac and skeletal muscle, and the role of the connective tissue network in cardiac and skeletal muscle, and the role of the connective tissue network in muscle atrophy and hypertrophy.

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Connective Tissue of Copper Deficient Animals


