Transgenic mice overexpressing both amyloid β-protein and perlecan in pancreatic acinar cells

K. Fukuchi1, M. Hart1, Z. Yan1, J.R. Hassell2 and L. Li3
Departments of 1Genetics and 3Medicine, Schools of Medicine and Dentistry, University of Alabama at Birmingham, Birmingham, Alabama and 2Shriners Hospitals for Children, Tampa, Florida, USA
Present addresses of Dr. Michael Hart is Auburn University, 311 Greene Hall Annex, Auburn University, AL 36849, USA

Summary. Heparan sulfate proteoglycans such as perlecan are thought to facilitate amyloid fibril formation. Tg3695 mice overexpress perlecan core protein in many tissues including the brain and pancreas. Tg13592 mice overexpress the signal plus 99-amino acid carboxyl terminal sequences (C99) of amyloid β-protein precursor in multiple tissues and develop amyloid deposits in the pancreas. To investigate a role of perlecan in β-amyloidosis, we established doubly transgenic mice by crossing the two lines of transgenic mice. The expression levels of the two transgenes remained unchanged in the brain and pancreas and the doubly transgenic mice did not develop amyloid deposits in the brain up to 19-months of age. Amyloid load detected by thioflavine S in the pancreas of the doubly transgenic mice was not significantly different from that in the transgenic littermates expressing only C99. Amyloid load in the pancreas increased during aging. We found a positive correlation between the Aβ-immunoreactive (non-fibrillar and fibrillar) and thioflavine S-positive (fibrillar) Aβ deposits in the single (C99) but not doubly transgenic mice. Our results suggest that perlecan does not independently influence amyloid formation in the pancreas of the transgenic mice and that there may be other factors that may modulate amyloid formation together with perlecan.

Key words: Amyloid, Perlecan, Transgenic Mice, Pancreas

Introduction

Amyloidosis is a generic diagnosis for a variety of diseases characterized by deposits of abnormal protein aggregates exhibiting certain common physical, and chemical features such as 8 to 10 nm thick and up to 100 nm long fibrillar, unbranched appearance on electron microscopy, and yellow-green fluorescence after thioflavine S staining, respectively. Amyloidosis is found in cancer, rheumatoid arthritis, Alzheimer disease (AD), chronic renal dialysis, familial amyloid polyneuropathy, spongiform encephalopathy and diabetes. Over 16 biochemically distinct proteins that form characteristic amyloid fibrils are known. It is likely that some components involved in amyloid formation, deposition and accumulation are not unique to only one type of amyloidosis, but play roles in the pathogenesis of other types of amyloid as well. A common mechanism may help explain why all different types of amyloid demonstrate common physical and chemical properties in spite of differences in amino acid sequences among amyloid forming proteins. Because heparan sulfate proteoglycan (HSPG) is the macromolecules consistently associated with all types of amyloid, HSPG is considered to be involved in such common mechanism (Snow and Wight, 1989). In addition, several lines of evidence suggest that HSPG play an important role in the etiology of amyloidosis: (i) heparan sulfate influences a specific amyloid precursor, apolipoprotein SAA, to form amyloid fibrils (McCubbin et al., 1988), (ii) in a mouse model for AA amyloidosis, perlecan (a specific HSPG) gene expression precedes amyloid formation (Ailles et al., 1993), (iii) both sulfate ions and heparan promote the aggregation of Aβ-fibril formation in vitro, which is a main constituent of amyloid in Alzheimer patients (Fraser et al., 1992), and (iv) in a rat infusion model for β-amyloidosis, HSPG is essential for persistence of amyloid deposits (Snow et al., 1994).

We and others demonstrated that overexpression of the signal plus amyloid β-protein (Aβ)-containing 99-amino acid carboxyl terminal sequences (C99) of its precursor under the control of β-actin promoter consistently led to ~10 nm amyloid fibrils in acinar cells of pancreas (Kawarabayashi et al., 1996; Fukuchi et al., 2000). In spite of sizeable overexpression of C99 in the brain, however, our transgenic mice (Tg13592) did not develop amyloid deposits in the brain (Fukuchi et al.,...
We also established a line of transgenic mice (Tg3695) that overexpressed ~400 kDa entire protein of perlecan in the brain, pancreas, and other tissues (Hart et al., 2001). Tg3695 mice, however, did not develop amyloid deposits in the tissues including the brain and pancreas (Hart, 2001).

In order to study the roles of perlecan and C99 in β-amyloidosis, we have established transgenic mice that expressed both C99 and perlecan by mating Tg13592 with Tg3695 mice. The effect of perlecan overexpression on amyloid formation in the doubly transgenic mice was determined by quantifying amyloid load in the pancreas. Since both perlecan and C99 were expressed in the brain of the doubly transgenic mice, possible amyloid deposits in the brain were also examined up to 19 months of age.

Materials and methods

Transgenic mice

Establishment, propagation, and maintenance of transgenic mouse lines, Tg13592 and Tg3695, were previously described (Fukuchi et al., 1996; Hart et al., 2001). All of the Tg13592 mice used in this study had been back-crossed to C57BL/6 mice more than 8 generations (B6.Tg13592[N9 to N11]). Tg3695 mice overexpress the entire perlecan core protein (~400 kDa) under the control of a cytomegalovirus enhancer/β-actin promoter. Tg3695 mice had been back-crossed to C57BL/6 more than 4 generations (B6.Tg3695[N5 to N7]) before breeding for production of doubly transgenic mice. Doubly transgenic mice (TgPerlecan/C99 mice) that overexpress both perlecan and C99 were produced by crossing Tg13592 with Tg3695 mice. Segregation of the transgenes was determined as described before (Fukuchi et al., 1996; Hart et al., 2001). Littermates carrying only either the C99 or perlecan transgene were used as controls and designated as TgC99 or TgPerlecan mice, respectively. Mice were monitored for the presence of murine pathogens by a comprehensive battery of virus serologies, bacterial cultures, endo- and ectoparasite examinations, and histopathology of all major organs, as described previously (Faulkner et al., 1995). All mice were consistently negative for pathogens by these tests. All animal protocols used for this study were prospectively reviewed and approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham.

Western blotting

Four 6-month-old mice from each of TgPerlecan/C99 and TgC99 mice were used to determine the steady state levels of C99 expression in the brain and pancreas by western blot analysis. The tissues were homogenized in 2x Laemmli buffer (1x = 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.001% bromophenol blue), boiled for 5 minutes, and sheared with 26-gauge needles. Protein concentration was determined by Protein Assay (Bio-Rad, Hercules, CA). Fifty µg of protein for each sample were applied to a 16.5% Tris-Tricine SDS-polyacrylamide gel electrophoresis (PAGE) and electrotransferred to polyvinylidine difluoride (PVDF) membranes (Immobilon-P, Millipore, Bedford, MA). The membranes were blocked with phosphate-buffered saline (PBS) containing 5% nonfat dried milk (w/v), 0.02% sodium azide, and 0.02% Tween 20, incubated at 4 °C for 16 hours with the 994B antibody and immunostained with an enhanced chemiluminescence system (Amersham, Arlington Heights, IL) according to the manufacturer’s protocol. The 994B antibody was developed against the 39 carboxyl-terminal residue of C99 (Fukuchi et al., 1996, 2000). The relative concentration of the protein was determined by densitometric scanning of the membrane using a Fluor-S Multilager and PDQuest software (Bio-Rad, Hercules, CA). A two sample t-test was used for statistical analysis.

Histochemical and immunohistochemical analyses

The numbers and ages of TgPerlecan/C99 and TgC99 mice used for detection of amyloid fibrils by thioflavine S and immunohistochemistry are listed in the Table 1. Mice were sacrificed by intraperitoneal injection of sodium pentobarbital. Major organs (brain, lung, heart, liver, spleen, pancreas, intestine, kidney, overly, testis and skeletal muscle) were removed and fixed in 10% formaldehyde: 90% alcohol followed by 10% buffered formalin. The tissues were stained with hematoxylin and eosin for evaluation of standard histology, thioflavine S for detection of amyloid and modified Bielschowsky for detection of neurofibrillary tangles (NFTs) (Yamamoto and Hirano, 1986). Immunohistochemistry was performed using a battery of

<table>
<thead>
<tr>
<th>GENOTYPE</th>
<th>AGE (months)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TgC99</td>
<td>1 1 3 1 1 2 3 1 1 3 5 1</td>
</tr>
<tr>
<td>TgPerlecan/C99</td>
<td>1 1 3 1 1 2 3 2 3 1 3 1</td>
</tr>
</tbody>
</table>
different primary antibodies and the Vectastain ABC kit (Vector, Burlingame, CA) as described below. The following primary antibodies were used: anti-perlecan (Klein et al., 1988) and HK-102 (Seikagaku, Falmouth, MA) for detection of perlecan core protein; HepSS-1 (Seikagaku) and 10E4 (Seikagaku) for detection of heparan sulfate glycosaminoglycan (HSGAG); Tau-2 (Boehringer Mannheim, Indianapolis, IN) for detection of tau; PHF-1 (Greenberg et al., 1992) for detection of paired helical filaments; 6E10 (Senetek, Maryland Heights, MO), 4G8 (Senetek), and anti-β-amyloid (Zymed, San Francisco, CA) for detection of Aβ.

For morphometric analysis, the fixed pancreas from each mouse was evenly cut into 6 pieces and embedded in one paraffin block with the caudal sides facing up so that each section will be separated more than 1.5 mm. The paraffin blocks were sectioned at 10 µm for thioflavine S staining and 5 µm for immunocytochemistry.

The 10 µm sections were stained with fresh, filtered, aqueous 1% thioflavine S for 5 min and differentiated with 70% ethanol for 5 min. After rinsing in distilled water twice, the sections were mounted in glycerin-H2O (3:1) and the images were immediately captured through a fluorescence microscope as described below.

The 5 µm-sections were subjected to the avidin-biotin immunoperoxidase method to detect Aβ-immunoreactive deposits using Vectastain ABC kit. Endogenous peroxidase was eliminated by treatment with 3% H2O2 for 30 minutes after deparaffinization of the sections. After washing with distilled water, sections were treated with 88% formic acid and rinsed with water and 0.1 M Tris-saline (TBS) (pH 7.4). Sections were blocked with 5-15% goat or horse serum in TBS for 60 minutes at room temperature and incubated with primary antibodies in 0.1 M TBS containing 5-15% serum (goat serum for rabbit antibodies and horse serum for mouse monoclonal antibodies) for 16 hours at 4 °C. Sections were rinsed in 0.1 M TBS containing 1% serum and incubated with appropriate biotinylated secondary antibodies for 60 minutes at room temperature. After washing, sections were incubated with Vectastain ABC reagent for 60 minutes at room temperature. Peroxidase activity was detected by treatment with 3,3' diaminobenzidine. The sections were counterstained with hematoxylin.

Morphometric and statistical analyses

Thioflavine S stained sections were visualized using a fluorescence Leica DMR with excitation wave lengths of 450-490 nm and emission of 515 nm under 10x PL Fluotar objective and images were captured through a Spot RT camera (Diagnostic Instruments, Sterling Heights, MI) as digitized tagged-image format files to retain maximum resolution. The images were quantified using image analysis software, Image-Pro Plus (Silver Spring, MD). A macro was written to measure the area of each object in the image. Artifacts, adipose tissues, islets of Langerhans, interlobular connective tissues, large blood vessels and ducts were manually excluded. The immunostained sections were observed under a Leica DMR light microscope (Wetzlar, Germany) with 10 x PL Fluotar objective and the images were captured and processed as described above.

The data were transferred to Microsoft Excel for tabulation. The measurements included total area (mm²) of 6 sections from each mouse (excluding artifacts, adipose tissues, islets of Langerhans, interlobular connective tissues, large blood vessels and ducts), thioflavine S positive area, and Aβ-immunoreactive area. Amyloid load (amyloid fibril formation) was calculated as the percentage of area showing thioflavine S fluorescence for each mouse. A two sample t-test was used to assess for a statistical difference in amyloid load between TgPerlecan/C99 and TgC99 mice.

A least squares linear regression analysis was used to evaluate for an increase in amyloid load with aging in both TgPerlecan/C99 and TgC99 mice. The association between thioflavine S positive and Aβ-immunoreactive deposits was studied by the least squares linear regression analysis, also.

Results

Levels of C99 expression in the pancreas and brain of TgPerlecan/C99 mice

Using 16.5% Tris-Tricine SDS-PAGE and the 994B antibody, 9 to 14 kDa fragments of C99 were observed in the pancreas (Fig. 1A) and brain (Fig. 1B) of both TgPerlecan/C99 and TgC99 mice by western blotting and quantified by densitometric scanning of the membrane. The steady state levels of C99 in the pancreas and brain of TgPerlecan/C99 mice were not different from those in TgC99 mice (p=0.67 and p=0.44, respectively). Thus, overexpression of perlecan did not influence the steady state levels of C99 in TgPerlecan/C99 mice.

Remarkable immunoreactivity for the perlecan core protein by anti-perlecan antibody or HK-102 in TgPerlecan/C99 mice was observed in almost all acinar cells of the pancreas and islets of Langerhans (Fig. 2A,B) while immunoreactivity for the perlecan core protein in non-transgenic mice was predominantly limited to the extracellular matrix and basement membranes (Fig. 2D). The intensity and pattern of perlecan immunoreactivity in TgPerlecan/C99 mice were similar to those in TgC99 mice (Fig. 2C). Thus, overexpression of C99 seems not to influence the expression of the perlecan transgene in TgPerlecan/C99 mice.

Aβ immunoreactive granular to globular deposits occurred mostly in acinar cells of the pancreas in TgPerlecan/C99 mice (Fig. 2E,F). A similar pattern for Aβ immunoreactivity was seen in TgC99 mice (Fig. G). No Aβ immunoreactivity was found in non-transgenic littermates (Fig. 2H). A similar patter for Aβ...
immunoreactivity between a rabbit polyclonal antibody (anti-ß-amyloid) and mouse monoclonal antibodies (6E10 and 4G8) except for an occasional staining in the blood vessels. The staining in the blood vessels by the mouse monoclonal antibodies is thought to be nonspecific to Aß but due to the anti-mouse IgG secondary antibody. Therefore, the staining in the blood vessels was excluded from quantitative analysis.

The pattern for Aß immunoreactivity appeared to be different from that for perlecan immunoreactivity in the pancreas of TgPerlecan/C99 mice (Fig. 2B,F). Thioflavine S and modified Bielschowsky staining did not reveal any amyloid deposits and NFT in the brain of TgPerlecan/C99 mice up to 19-months of age. Furthermore, immunohistochemistry performed with various antibodies against Aß, tau and PHF did not yield significant differences between TgPerlecan/C99 and TgC99 mice. The tissues evaluated were those in which amyloid deposits are prone to occur (brain, heart, liver, lung, pancreas, kidney, intestine, muscle and spleen).

Aging dependent Amyloid load in the pancreas

Previously we reported that Tg13592 mice developed ~10 nm amyloid fibrils in the cytoplasm of pancreatic acinar cells and that amyloid can be detected by thioflavine S (Fukuchi et al., 2000). We quantified amyloid load in the pancreas of both TgC99 and TgPerlecan/C99 mice by thioflavine S staining to evaluate for a possible correlation between amyloid load and aging. The mean area examined was 32.45±2.50 and 34.29±2.55 mm²/mouse for TgC99 and TgPerlecan/C99 mice, respectively. The percentage of area showing thioflavine S fluorescence for each mouse is plotted in Figure 3A. A positive correlation (correlation coefficient, \( r=0.79, p<0.001 \)) between aging and amyloid load was found for the C99 mice (Fig. 3B). Amyloid load also increased during aging of TgPerlecan/C99 mice, also \( (r=0.78, p<0.001) \) (Fig. 3C).

No difference in amyloid load between TgPerlecan/C99 and C99 mice

In order to study the influence of perlecan on amyloid formation, the percentage of thioflavine S positive area in the pancreas of TgPerlecan/C99 mice was compared with that of TgC99 mice. The mean % of areas showing fluorescence (1.43±0.30%) in TgPerlecan/C99 mice was not significantly different from that in TgC99 mice (1.66±0.35; \( p=0.31 \)). For the following statistical analyses, we have divided the mice into two age groups, 6- to 11-month-old and 14- to 19-month-old, because, as we reported previously, approximately 50% of 3- to 6-month-old Tg13592 mice developed Aß-immunoreactive deposits and because Tg13592 mice older than 12 months consistently had substantial amyloid fibrils readily detectable by thioflavine S in the pancreas (Fukuchi et al., 2000). The mean percentages of thioflavine S positive areas in the 6- to 11-month-old mice were 0.592±0.106 and 0.661±0.134 for TgPerlecan/C99 and TgC99 mice, respectively. There is no difference between the two
Fig. 2. Comparative expression of perlecan and Aβ by immunohistochemistry in the pancreas of TgPerlecan/C99 (A, B, E, and F), TgPerlecan (C), TgC99 mice (G), and non-transgenic littermates (D and H) and fibrillar Aβ detected by thioflavine-S (I and J). A-D. Perlecan was visualized by anti-perlecan core protein antibody. TgPerlecan/C99 and TgPerlecan transgenic mice similarly show conspicuous immunoreactivity to perlecan in the extracellular matrix and the cytoplasm of the acini and cells of the islets of Langerhans (A and B: 9-month-old TgPerlecan/C99 mouse; C: 9-month-old TgPerlecan mouse) while immunoreactivity in a non-transgenic littermate is limited to the extracellular matrix (D). E-H. Aβ deposits were detected by rabbit polyclonal anti-β-amyloid antibody. Accumulation of Aβ deposits is seen in the cytoplasm of most acinar cells in both TgPerlecan/C99 (E and F) and TgC99 (G) mouse. No pancreatic tissue staining is seen in the non-transgenic littermate (H). I and J. Fibrillar Aβ demonstrating thioflavine-S fluorescence in acinar cells of the pancreas in a 14-month-old TgPerlecan/C99 mouse. B, F, and J are higher magnifications of A, E, and I, respectively. Bars: A, C-E, G, H, 50 µm; B, F, 10 µm; I, 70 µm; J, 20 µm.
groups ($p=0.38$). The mean % of areas showing fluorescence (2.54±0.45%) in the 14- to 19-month-old TgPerlecan/C99 mice was not significantly different from that in the 14- to 19-month-old TgC99 mice (3.04±0.46%; $p=0.27$), also.

Aβ-immunoreactive amyloid deposits can exist in two forms: 6-10 nm amyloid fibrils showing fluorescence by thioflavine S staining and non-fibrillar aggregates of Aβ. Because perlecan is thought to influence the transition of amyloid deposits (non-fibrillar to fibrillar form), we studied a correlation between Aβ-immunoreactive amyloid deposits and thioflavine S positive amyloid deposits. Scatterplots of the percentage of area showing thioflavine S fluorescence versus that for Aβ-immunoreactivity are shown in figure 4. There was a positive correlation between the percentage of the positive area for thioflavine S and that for Aβ-immunoreactivity in TgC99 mice ($r=0.69, p<0.02$) while no correlation was found in TgPerlecan/C99 mice ($r=0.322, p>0.1$).

**Discussion**

We have produced doubly transgenic mice that overexpress both C99 and perlecan in multiple tissues including the brain and pancreas. Because Tg13592 and Tg3695 mice showed robust expression of C99 and perlecan in the pancreatic tissues, respectively, and Tg13592 mice developed amyloid deposits within acinar cells of the pancreas, we expected an increase in amyloid formation in the pancreas of TgPerlecan/C99 mice. Expression of the C99 transgene was not influenced by that of the perlecan transgene in the TgPerlecan/C99 mice compared with TgC99 mice and expression of the perlecan transgene vice versa compared with TgPerlecan mice. Our results suggest that overexpression of both
perlecan and C99 does not result in a synergistic effect on Aβ fibril formation.

Aβ deposits in most cases of amyloidosis occur extracellularly. On the other hand TgPerlecan/C99 showed remarkable expression of intracellular Aβ and intracellular perlecan. Lack of the Aβ and perlecan accumulation in the extracellular matrices may be one possible reason why TgPerlecan/C99 mice failed to show the synergistic effect by overexpression of both perlecan and C99 on amyloid fibril formation. An other possible explanation is that HSGAGs on overexpressed perlecan core protein in the transgenic mice exist, possibly, shorter or in small numbers, and therefore, at low levels indistinguishable by immunocytochemistry. Previous cell culture work using an expression vector containing only a portion (domains 1, 2, and 3) of the perlecan cDNA (domain 1 being the region known to be glycosylated) showed that a substantial amount of the recombinant product was produced without HSGAG side chains (Dolan et al., 1997) and that overexpression of perlecan domains 1 and 2 produced perlecan with ~12 kDa HSGAG chains instead of the usual 70 kDa HSGAG chains, indicating that the overexpression of perlecan core protein was not coupled with an increase in HSGAG chain synthesis (Doege et al., 1997). Perlecan binds amyloid proteins, accelerates Aβ fibril formation, and maintains Aβ fibril stability. Most of these effects of perlecan have been shown to be due to its associated HSGAG side chains (Snow and Wight, 1989; McCubbin et al., 1988; Fraser, 1992; Castillo et al., 1997, 1999). Thus, an obvious increase in HSGAGs may be an essential step for the induction of amyloidosis. The lack of an increase in HSGAGs may be one possible reason for our failure in demonstrating such effects of perlecan on amyloidosis. Kawarabayashi et al. (1996) also established transgenic mice overexpressing C99 and reported that Aβ amyloid fibrils accumulated within lysosomes of pancreatic acinar cells in their transgenic mice. It is most likely that the Aβ immunoreactive deposits in TgPerlecan/C99 mice accumulated in lysosomes of pancreatic acinar cells because of close similarities between DNA constructs, as well as immunocytochemical and transmission electron microscopic findings in the two transgenic mice (Kawarabayashi et al., 1996; Fukuchi et al., 2000). On the other hand, perlecan expression appeared to be broadly distributed in the cytoplasm of pancreatic acinar cells in TgPerlecan/C99 and Tg3576 mice. Therefore, it is possible that Aβ aggregates were sequestered from overexpressed perlecan in the mice.

A positive correlation between Aβ-immunoreactive and thioflavine S positive deposits was found in TgC99 mice but not in TgPerlecan/C99 mice. The reasons for this discrepancy are not clear. This data, however, suggests that there may be a factor(s) in pancreatic acinar cells, which modulate(s) formation and stability of amyloid fibrils in combination with overexpressed perlecan core protein. Because TgPerlecan/C99 mice were not on a pure C57BL/6 background, expression of such factors may vary based upon the differences in genetic background among TgPerlecan/C99 mice. Such factors can be enzymes involved in glycosylation of perlecan. Therefore, TgPerlecan/C99 mice may be useful for identification of such factors.

Recently, intracellular Aβ and its aggregates have emerged as possible causative agents in the pathogenesis of AD and several investigators proposed reducing intracellular Aβ as an efficient therapeutic target (Bayer et al., 2001; Klein, 2002; Walsh et al., 2002; Selkoe, 2002). However, they are difficult to study in AD brain and other model systems. On the other hand, accumulation of intracellular Aβ in Tg13592 and TgPerlecan/C99 mice was readily detectable and no extracellular amyloid deposit was observed. Thus, these transgenic mice may serve as unique model systems for the investigation of the mechanisms engendering intracellular Aβ, its deposition and clearance as well as screening tools for therapeutically means against β-amyloidosis.

In summary, we have established transgenic mice that overproduce both perlecan core protein and C99. The transgenic mice did not develop extracellular amyloid deposits or intracellular tangles. No synergistic effect of perlecan and C99 on β-amyloidosis was found in the transgenic mice. There was a positive correlation between Aβ load detected by an antibody against Aβ and that detected by thioflavine S fluorescence in TgC99 mice but not in TgPerlecan/C99 mice. These transgenic mice will be useful to identify additional factors that modulate amyloid fibril formation together with perlecan.

Acknowledgements. We thank A.D. Snow, J.R. Lindsey and J.M. Wyss for useful discussion, T.K. Hinds and K. Kamino for 994B antibody, P. Davies for PHF-1 antibody, L.E. Harrell and G. Zhang for AD brain tissues through Alzheimer's Disease Center, University of Alabama at Birmingham, A. Smith, T. Gunn and R. Kawai for technical assistance. This research is supported in part by the National Institute of Health (AG12850, NS43947, RR11105, and RR07003).

References
Doege K., Chen X., Cornuet P.K. and Hassell J. (1997). Non-
glycosaminoglycan bearing domains of perlecan and aggrecan influence the utilization of sites for heparan and chondroitin sulfate synthesis. Matrix Biol. 16, 211-221.


Accepted March 30, 2004