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Expression of Laminin and Type IV Collagen by Basement Membrane-Producing EHS Tumors in Streptozotocin-Induced Diabetic Mice: *In Vivo* Modulation by Low-Molecular-Weight Heparin Fragments

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ABSTRACT. The biosynthesis of basement membrane components in Engelbreth Holm Swarm-bearing mice with or without streptozotocin-induced diabetes and the effect of low-molecular-weight heparin derivatives (CY222, Sanofi Recherche/Institut Choay) on the relative rates of these synthetic activities were studied. In diabetic mice, the laminin mRNA level increased, whereas type IV collagen mRNA decreased. *In vivo* treatment with heparin fragments decreased the mRNA level of laminin to control values without altering the mRNA level of collagen IV. Biosynthetic studies with radiolabeled precursors ([^{3}H]-proline for collagen and [^{35}S]-methionine for laminin) confirmed these results. Laminin protein biosynthesis increased in diabetic mice. Treatment with CY222 corrected this alteration. Our results suggested an increased labeling of polymeric forms of collagen IV in diabetic mice. In addition, we showed that biosynthesis of acid-extractable collagen IV decreased in diabetic mice and that CY222 treatment corrected this disturbance. These experiments suggest that low-molecular-weight heparin fragments CY222 can modulate the biosynthesis of extracellular matrix macromolecules altered in diabetic animals by different pathways, including pretranslational and posttranslational steps. *Copyright © 1996 Elsevier Science Inc.* BIOCHEM PHARMACOL 52;11: 1695–1701, 1996.

KEY WORDS. laminin; collagen IV-EHS tumor; basement membrane; diabetes; heparin

Basement membranes are specialized extracellular matrices, that separate cell layers from the underlying connective tissue. These membranes are implicated in embryogenesis and development, where they provide substrate for cellular adhesion and differentiation and act as a selective barrier to filtration [1, 2].

Basement membranes contain mainly type IV collagen [1, 2], glycoproteins as laminin [3] and nidogen/entactin [4], and heparan sulfate proteoglycan (perlecan) [5–7]. Type IV collagen and laminin are the predominant structural elements of basement membrane, where each macromolecular component constitutes a self-assembling network [1, 2]. These networks interact with other minor components of basement membranes. The basic structure of basement membrane can also be considered as a fine network of type IV collagen filaments, with the other basement membrane components, such as laminin, nidogen and heparan-sulfate proteoglycan, regulating pore size and thickness of the collagen network [8]. The ratio of these constituents plays a crucial role in the definition of functional qualities of basement membranes such as selective permeability. The ratio of these components is modified during aging [9, 10] and in diabetes [11]. Diabetic microangiopathy and aging are characterized by thickening of the basement membranes in different tissues, accompanied by deregulations of the biosynthesis of extracellular matrix macromolecules [11–15].

We reported on the biosynthesis of fibronectin and of collagen type III, which increased in explant cultures of diabetic KK mice skin [16]. These biosynthetic alterations were corrected by *in vivo* treatment with low-molecular-weight heparin fragments (CY222) [17]. These heparin fragments were also capable of modulating the biosynthesis of fibronectin and collagen type III by smooth muscle cells in culture [18]. This regulation concerned the transcriptional level [19]. In the present study, we wished to extend these experiments to the two major basement membrane components, collagen type IV and laminin.

The murine EHS[‖] tumor provides a model for in vivo

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‖ *Abbreviations:* EHS, Engelbreth Holm Swarm; DMEM, Dulbecco's modified Eagle's medium; PMSF, phenylmethylsulfenyl fluoride; MOPS, morpholmepropanesulfonic acid.

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studies of the biosynthesis of these components [3, 4]. This tumor can be transferred by cell suspension in control or in experimentally pathologic animals and provides sufficient quantities of basement membrane components for quantitative biochemical studies [20–24].

We report the *in vivo* effects of CY222 on collagen type IV and laminin mRNA steady-state and protein levels in EHS tumor-bearing control and streptozotocin-diabetic mice.

MATERIALS AND METHODS

Experimental Animals

Three-month-old male C57BL/6J mice were used in this study. Diabetes was induced by intraperitoneal injections of 135 mg/kg of streptozotocin in 0.1 M citrate buffer, pH 4.5 [23]. During these experiments, the animals were not treated with insulin. Blood glucose was determined before each experiment by drawing blood from the eye using sodium citrate buffer as anticoagulant. EHS tumor cell suspension was injected intramuscularly into the hind limbs, and the tumors were harvested after 2 weeks [20]. For diabetic mice, the EHS tumor was injected 3 weeks after the onset of diabetes. Injections of 2.7 mg/kg CY222 were done 2 weeks after the onset of diabetes and prolonged for 3 weeks at the rate of one daily injection 5 days per week. Control groups (non-CY222-treated normal and diabetic groups) were injected with isotonic saline solution. In all groups, the development of tumor was examined daily, and no apparent alteration of skin could be detected until the nonnecrotic tumors were harvested (2 weeks after tumor cell injection as described in [20]). In each group, all data are the mean (±SD of the mean) of eight determinations obtained from eight separate animals.

The schematic diagram of the experimental procedure is depicted below:

Diabetes induction		CY222 treatment	EHS injection		Tumor harvesting
0	1	2	3	4	5 (weeks)

Low-molecular-weight heparin fragments, CY222, average Mr:2500 D (Sanofi/Choay-France) were obtained as previously described [25].

RNA Analysis

RNA ISOLATION. Total RNAs were extracted from EHS tumor fragments (~1 g, frozen to −180°C) by guanidinium isothiocyanate, as described by Chomczinsky and Sacchi [26]. For quantitative assessments, aliquots of 2, 4 and 6 μg of total RNA were heat denatured at 65°C for 15 min in a 50% formamide, 6% formaldehyde, 0.2 M MOPS, 0.05 M sodium acetate, pH 7, and 0.01 M EDTA buffer. Total RNA was transferred to nylon filters (HYBOND N+, Amersham France) by using a commercial slot blot apparatus (Minifold, Schleicher & Schull, Dassel, Germany).

cDNA PROBES. The following cDNA probes were used: pHT21, containing a 2.6-kb insert coding for the human a1 (IV) collagen [27]; pHL40 specific for the human laminin B1 chain (3.6-kb insert) [28]; pH210, containing a 4-kb insert coding for human laminin B2 [29]; and pAL41, a mouse b actin probe [30] used to evaluate cellular activity.

Probes were labeled with [α^{32}P] dCTP (110 TBq/mmole, Amersham, France) by a Nick translation kit (Boehringer Mannheim) to specific activities (3–5.10^8 cpm/mg of DNA).

The specificity and integrity of the probes used in this study was determined by Northern blot analysis as described in [19] (see Fig. 1b).

SLOT-BLOTTING PROCEDURES. Prehybridization was done at 42°C for 24 hr in a buffer containing 50% (v/v) formamide, 5× SSPE (1× SSPE: 180 mM NaCl, 10 mM Na_2HPO_4, 1 mM EDTA, pH 7.4), 0.5% (w/v) SDS, 0.1% each of Ficoll, polyvinylpyrrolidone and bovine serum albumin and 200 μg/mL of heat-denatured sonicated salmon DNA. Hybridization was carried out in fresh buffer for 24 hr at 42°C. The filters were washed at 42°C with 2× SSPE, 1× SSPE, 0.1× SSPE and sequentially with 0.1% SDS. The dried filters were exposed to X-ray films at −80°C. The autoradiograms were quantified by densitometric scanning with a laser densitometer (Ultrascan 2202 LKB, Integrator 2220 LKB). For hybridization with control actin probe, membranes previously hybridized with specific probes (for laminin or collagen) were dehybridized and then rehybridized with beta actin probe.

Protein Studies

After 2 weeks, EHS tumors were harvested for incubation with labeled amino acids for biosynthetic studies.

LAMININ BIOSYNTHESIS. Three grams of the tumor were minced and rinsed several times with DMEM lacking methionine. Tumor fragments were then incubated at 37°C for 4 hr in 15 mL of methionine-free DMEM containing 2.8 MBq of L-[^{35}S]-methionine (specific activity, 37 TBq/mmol, Amersham, France). Laminin was essentially contained in the 0.5-M NaCl extract (0.05 M Tris/HCl, pH 7.4, containing 0.5 M NaCl, 0.5 mM PMSF, 0.5 mM ethylmaleimide and 10 mM EDTA), as described by Timpl *et al.* [21]. Extensive extraction of the residual tissue with the same buffer containing 3.4 M NaCl provided less than 1% immunoprecipitable radiolabeled laminin.

Laminin was characterized by immunprecipitation from aliquots of the 0.5-M NaCl extract with IgG from a rabbit anti-mice EHS laminin serum (Institut Pasteur, Lyon, France). Specificity of immunoprecipitation was controlled by Western blot (Fig. 2). Laminin biosynthesis is evaluated by the percentage of radioactivity recovered in immunoprecipitate relative to total radioactivity incorporated in total minced tissue proteins (expressed as dpm/mg protein).

COLLAGEN TYPE IV BIOSYNTHESIS. Three grams of the tumor were minced, rinsed and incubated at 37°C for 4 hr

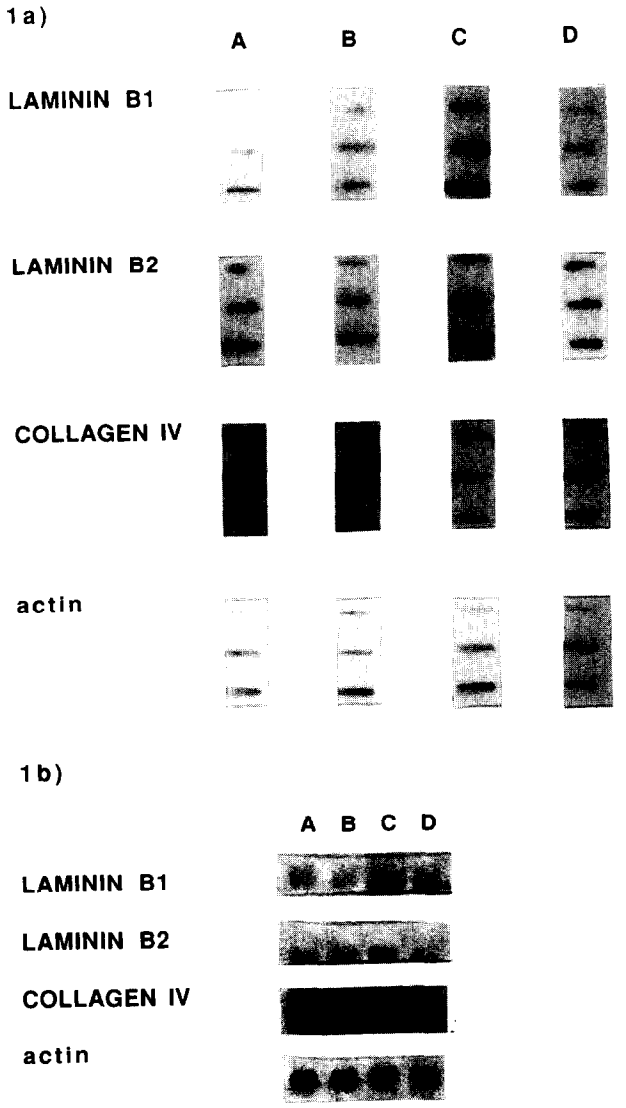

FIG. 1. (a) Slot blot and (b) Northern blot analyses of actin, laminin B1 chain, laminin B2 chain and alpha1 (IV) collagen mRNA levels in EHS tumor across groups. (a) Total RNA from tumor was extracted, and increased quantities (2, 4 and 6 μg) were applied to the filter and hybridized with [^{32}P]-labeled specific probes (as described in Materials and Methods). (b) Northern blot analysis of mRNAs: laminin B1, 5.6 kb; laminin B2, 7.6 kb; alpha1 (IV), 6.9 kb; and beta actin, 2.1 kb. (A) Control mice, (B) CY222-treated control mice, (C) diabetic mice and (D) CY222-treated diabetic animals.

in 15 mL of DMEM containing 0.74 MBq/ml of L-[2,3,4,5 ^{3}H] proline (specific activity, 4 TBq/mmol, Amersham, France); β-aminoproprionitrile (50 μg/ml) and ascorbic acid (50 μg/mL), supplemented with 10% of fetal calf serum. Extraction was performed as described for tissular collagen [16] and EHS tumor [21], with some modifications. Briefly, EHS tumor fragments were washed with phosphate buffered saline, homogenized in ice-cold 0.5 M acetic acid (5 mL/g tumor) containing 10 mM EDTA and 4 mM ethylmaleimide and extracted for 24 hr. The suspension was clarified by centrifugation (25,000g for 30 min at 4°C). The supernatant contained acid-extracted type IV collagen (acid-soluble collagen fraction). The undissolved material present in the pellet was rehomogenized and solubilized by digestion with 0.5 mg/mL of pepsin in 0.5 M acetic acid for 48 hr (Sigma Chimie, France). This suspension was centrifuged, and the supernatant-containing pepsin-soluble collagen was concentrated by precipitation with 2 M NaCl, dissolved in acetic acid and then dialyzed.

This pepsin-soluble fraction contained a polymerized form of collagen (pepsin-insoluble residue containing less than 5% of total EHS collagen was discarded). For studies

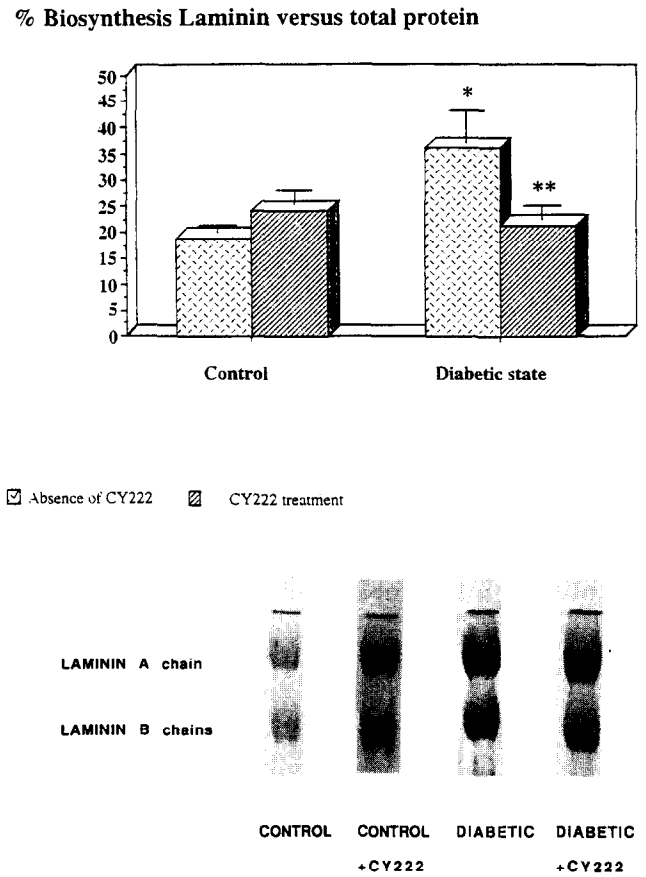

FIG. 2. Biosynthesis of laminin in control and diabetic mice; effects of CY222 treatment. Bar graph: EHS tumor was labeled with [^{35}S]-methionine, and laminin was extracted from tissues and immunoprecipitated as described in Materials and Methods. Biosynthesis of laminin was expressed as [^{35}S]-methionine incorporated in immunoprecipitated laminin related to total labeling in tumor proteins (evaluated as dpm/mg protein). The values are the mean (±SD of the mean) of eight determinations. *$P < 0.001$ (diabetic vs. control) and **$P < 0.001$ (diabetic vs. diabetic + CY222). Blots: Typical laminin immunoprecipitation obtained with 0.5-M NaCl extract (no detectable immunoprecipitate was recovered in 3.4 M NaCl fraction or in culture medium). Total proteins underwent SDS-PAGE (7% acrylamide, 1% mercaptoethanol) and then immunodetected by immunotransfert. All groups exhibited a similar qualitative profile, although a detectable increase in laminin immunoprecipitate could be observed in diabetic mice.

of collagen biosynthesis, total hydroxyproline, hydroxy [^{3}H]-proline and [^{3}H]-proline were determined as previously described [16, 17].

Statistical analyses were performed with Student's t-test. For each group, all experimental values are the mean of eight determinations ± SD of the mean performed on eight separate animals.

DNA content of EHS tumor was determined by the Burton procedure [16].

RESULTS

Physiological Parameters of the Different Mice Groups

Determination of blood glucose levels indicated significant hyperglycemia in diabetic vs. mice. CY222 did not significantly modify glycemia in control animals or hyperglycemia in diabetic mice. The following values were obtained (expressed as mg glucose/mL): 1.15 (±0.4) for control mice and 1.26 (±0.5) for control + CY222, 3.10 (±0.8) for diabetic mice and 2.50 (±0.9) for diabetic + CY222 ($P < 0.001$ as compared with the control value).

Total DNA content of EHS tumors in the different groups was not significantly different. Values (expressed as μg/g tumor dry weight) were 10.6 (±3.5), 8.9 (±3.7), 10.6 (±3.8) and 10.1 (±3.5) for control, control + CY222, diabetic and diabetic + CY222 groups, respectively.

Effect of Heparin Fragments CY222 on Type IV Collagen and Laminin mRNA Levels

Figure 1a shows typical slot blot analysis of mRNAs extracted from EHS tumors from the different groups. Analytical Northern blots performed with the same mRNA extracts did not exhibit significant alteration of the samples, as judged by the respective molecular size of each probe (Fig. 1b). When specific mRNAs were quantified by densitometric analysis of slot blots, we obtained a linear relationship across the amounts of RNA applied to the filter for hybridization.

Quantitative densitometric analysis of these data are shown in Table 1. For each probe, the results are expressed as arbitrary units, defined as the intensity of the probe signal vs. the relatively constant level of actin mRNA.

In Table 1, we demonstrate that mRNA levels of both B1 and B2 laminin chains are significantly increased in EHS tissue from the diabetic group. Treatment of diabetic mice with CY222 restored B1 and B2 laminin mRNA levels to control values. In contrast, type IV mRNA levels decreased in diabetic mice, and no modification was obtained by treatment of diabetic mice with CY222. CY222 had no significant effect on laminin and collagen IV mRNA levels in control mice.

Effect of CY222 on Laminin and Type IV Collagen Biosyntheses

Biosynthesis of laminin was determined by specific immunoprecipitation from total EHS protein labeled with [^{35}S]-methionine (Fig. 2). Total protein synthesis, expressed as [^{35}S]-methionine dpm × 10^5/mg tumor proteins, was not significantly different across experimental groups: 5.4 (±0.7), 4.8 (±0.6), 6.0 (±0.7) and 5.3 (±0.6) for control, control + CY222, diabetic and diabetic + CY222 groups, respectively. Diabetic mice exhibited a significant increase in laminin biosynthesis in EHS tissue. Treatment of diabetic animals with CY222 decreased this alteration toward control values (Fig. 2, bar graph). Because total protein synthesis was quite similar in all groups, variations in laminin biosynthesis could be considered as absolute.

No qualitative differences in the laminin immunoprecipitation profile were observed across groups (Fig. 2, blots). However, we did observe a relative increase in immunoprecipitated laminin in diabetic mice, reflecting increased laminin biosynthesis.

Biosynthesis of type IV collagen was determined after incubation of EHS tissue with [^{3}H]-proline by extracting collagen with 0.5 M acetic acid (acid-soluble collagen) and then by pepsin digestion of polymerized labeled collagen (pepsin-soluble collagen). Table 2 shows the chemical amount of collagen (hydroxyproline content) in each fraction for the different groups. No significant variation could be noticed either in total collagen amount or in collagen distribution of fraction, except for a significant increase in pepsin-soluble collagen and total collagen in CY222-treated diabetic mice. In diabetic mice, hydroxyproline-specific radioactivity of acid-soluble collagen IV significantly decreased without alteration of this parameter in polymerized (pepsin soluble) collagen (Fig. 3A, B). CY222 treatment of diabetic mice restored the specific radioactivity of acid-soluble collagen to control values. This treatment also increased the hydroxyproline-specific radioactivity of pepsin-soluble collagen fraction in diabetic mice.

TABLE 1. Quantitative comparison of laminin B1, laminin B2 and alpha1 (IV) collagen mRNA levels

Probes	Control	Control + CY222	Diabetic	Diabetic + CY222
Laminin B1	0.25 (±0.03)	0.31 (±0.05)	0.61 (±0.08)*	0.22 (±0.03)**
Laminin B2	0.72 (±0.10)	0.93 (±0.15)	1.75 (±0.22)*	1.13 (±0.14)**
Alpha1 (IV)	1.66 (±0.21)	2.08 (±0.33)	0.86 (±0.12)*	1.10 (±0.13)

Data were obtained from densitometric analysis of Fig. 1a. Results are expressed as arbitrary units that correspond to the ratio of the density of each mRNA indicated in Fig. 1 to the density of actin mRNA. Data are the mean (±SD of the mean) of eight determinations on eight separate animals.

* $P < 0.001$ (diabetic vs. control); ** $P < 0.001$ (diabetic vs. diabetic + CY222).

TABLE 2. Collagen content (hydroxyproline) of acid-soluble and pepsin-soluble fractions from EHS tumor

	Control	Control + CY222	Diabetic	Diabetic + CY222
Acid soluble	32.8 (±5.5)	22.4 (±4.2)	28.5 (±4.5)	25.4 (±6.7)
Pepsin soluble	83.8 (±6.4)	93.8 (±5.9)	81.3 (±8.1)	133 (±17.5)*

Hydroxyproline was estimated on aliquot of each fraction as described in Materials and Methods and expressed as μg/g tumor (wet weight). Less than 5% of total tumor hydroxyproline could be detected in pepsin-insoluble residue. Data are the mean (±SD of the mean) of eight determinations on eight separate animals.

* $P < 0.001$ (diabetic + CY222 vs. control or diabetic).

If we consider the distribution of newly synthesized labeled collagen (Table 3), a significant increase in labeling incorporation in the polymerized collagen (pepsin-soluble collagen) in diabetic EHS tumoral tissue was observed. CY222 did not correct this alteration in diabetic mice.

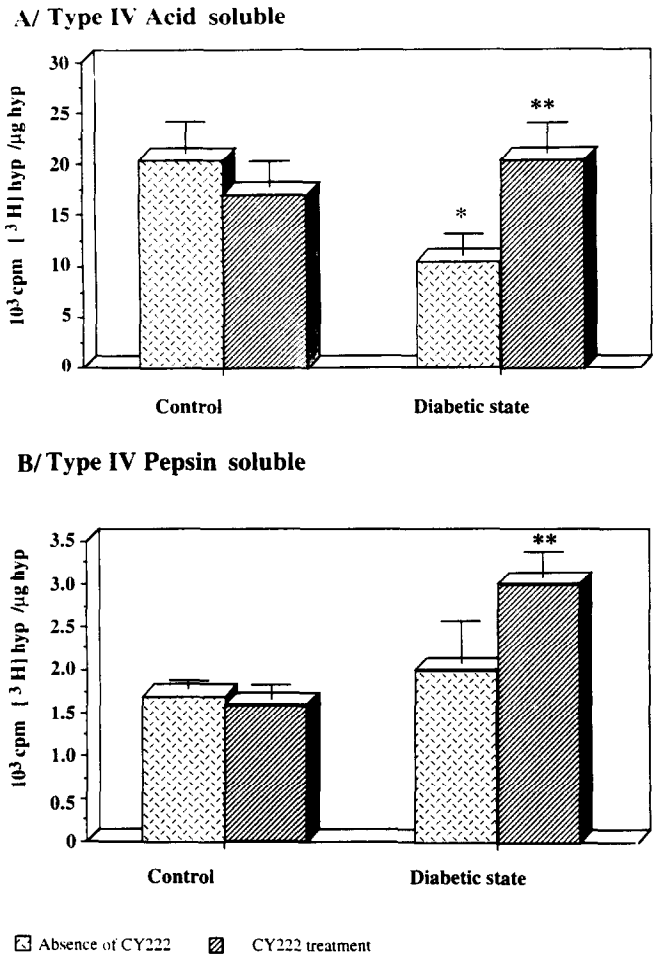

FIG. 3. Biosynthesis of collagen IV in control and diabetic mice; effect of CY222. EHS tumor was labeled with [³H]-proline; acid-soluble (A) and pepsin-soluble (B) collagen IV were extracted and the specific radioactivity of [³H]-hydroxyproline was determined in each collagen fraction as described in Materials and Methods. The values are the mean (±SD of the mean) of eight determinations. *$P < 0.001$ (diabetic vs. control) and **$P < 0.001$ (diabetic vs. diabetic + CY222).

DISCUSSION

Basement membrane thickening is the most prominent and characteristic feature of diabetic microangiopathy and it accompanies physiological aging. Data concerning alterations of basement-membrane-specific components during diabetes are often contradictory or unclear. It has been suggested that thickening is due to accumulation of basement membrane material deposited by successive layers of cells [31]. Others have proposed a decreased susceptibility of diabetic basement membrane to degradation [32]. Laminin B1 chain mRNA and collagen IV mRNA were increased in streptozotocin-diabetic rat glomeruli [33]. However, experimental diabetes in rats failed to show increased laminin and collagen IV mRNA levels in retina [34], whereas increased expression of collagen IV mRNA was detected in human diabetic retinopathy [14]. These apparent discrepancies could be attributed to differences in experimental models. Furthermore, the role of hyperglycemia and glycation products in the upregulation of basement membrane components should also be considered [35]. In this study, we used EHS basement membrane transplanted to streptozotocin-diabetic mice. Under our experimental conditions, we could show a significant increase in laminin biosynthesis (Fig. 2), which correlated well with an increased steady state level of mRNA coding for laminin B1 and B2 chains (Table 1, i.e., 2.0-fold increased laminin protein synthesis vs. an approximate 2.4-fold increase in laminin mRNAs levels).

Ours results suggest that overexpression of laminin in diabetic EHS basement membrane implicate pretranslational mechanisms. Similar variations in laminin expression were reported in streptozotocin-diabetic rat glomeruli [33] and in human diabetes [36]. The significant hyperglycemia present in these models, as in our diabetic mice, may play a role in this process, as suggested by the high-glucose-induced overexpression of basement membrane components [15, 37, 38].

However, we demonstrated that *in vivo* treatment of diabetic mice with low-molecular-weight heparin fragments (CY222) do not significantly decrease hyperglycemia in diabetic mice, although this treatment restored both laminin mRNA levels and laminin biosynthesis to control values. These results argue for a direct action of heparin fragments on laminin biosynthesis at the pretranslational level.

TABLE 3. Percentage of [^{3}H]-hydroxyproline incorporated in pepsin-soluble collagen to total incorporated [^{3}H]-hydroxyproline (acid- + pepsin-soluble collagen)

	Control	Control + CY222	Diabetic	Diabetic + CY222
[^{3}H]-hydroxyproline	18 (±2)	23 (±3)	37 (±4)*	45 (±5)

Data are calculated as ([^{3}H]-(hydroxyproline in pepsin-soluble fraction × 100) ÷ [^{3}H]-hydroxyproline in acid + pepsin-soluble fractions. Data are the mean (±SD of the mean) of eight determinations from eight separate animals.

* $P < 0.01$ (diabetic vs. control).

As for collagen IV, we showed an apparent decrease in acid-soluble collagen synthesis in diabetic mice (Fig. 3A), which was correlated with a decrease in the collagen IV mRNA level (Table 1). Different and conflicting results were previously reported, indicating no difference in total collagen content in EHS tumor from streptozotocin-diabetic mice [23] and no change in collagen IV synthesis in retina from diabetic animals [34] but an increase in collagen IV synthesis in diabetic rat glomeruli [33] and in human diabetic retinopathy [14].

As previously described [23] with a similar streptozotocin-diabetic model, we did not observe a significant increase in total EHS tumor collagen in our diabetic mice (Table 2). However, if we consider the distribution of newly synthesized labeled hydroxyproline between acid-soluble collagen and polymeric (pepsin soluble) collagen (Table 3), we demonstrate a significant increase in polymeric collagen labeling in diabetic mice. A comparable increase in polymeric collagen formation was described in other diabetic models and in human diabetic tissues [16, 39, 40].

Heparin fragments do not alter any parameter of collagen biosynthesis in control mice. These fragments, when injected into diabetic mice, increase the labeling of both acid- and pepsin-soluble collagen fractions (Fig. 3) and as a consequence probably increase total collagen present in EHS tumor (Table 2). This increase essentially concerns pepsin-soluble fraction, suggesting a more polymerized form of collagen in the diabetic treated group. However, these modulations of collagen biosynthesis are not correlated with modulation in collagen mRNA level and probably implicate posttranslational steps. Activity of heparin and related compounds on polymerization of different collagen types has been reported [41, 42]. Heparin fragments may be more effective on diabetic mice tumor than on control mice tumor because of the relative disorganization of diabetic basement membrane tumor, as shown by laminin alteration presented in this report.

To conclude, heparin and its derivatives may exhibit pleiotropic activities on cells and extracellular matrix components [43] by using different mechanisms for different macromolecules, including pretranslational and posttranslational processes during basement membrane precursor biosynthesis.

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