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Ⅰ．招へい責任者　下門顕太郎

所属機関　国立循環器病センター研究所　職名　室長

所在地　〒565-8565 大阪府吹田市藤白台5丁目7番1号　電話　06-833-5012

招へい研究者氏名　宋志清

所属機関　泰安中心医院（中華人民共和国 山東省）

職　名　主治医師

Ⅱ．日本滞在日程

平成９年８月２８日来日、平成 10 年２月 25 日　帰国のため離日した。
この間、国立循環器病センター研究所の研修生の身分で、同研究所　機能評価研究室において、血管壁の細胞情報伝達に関する研究に従事した。成果は第 62 回日本循環器学会学術集会にて発表予定であり、また論文を投稿中である。

Ⅲ. 研　究　報　告（4000字以上で記入して下さい　別紙可）

和文抄録

血管傷害による血管壁内の細胞内情報伝達の変化。
－とくに接着斑のチロシンりん酸化蛋白の変化について－

【目的】最近、細胞と細胞外マトリックスの接着部位(focal adhesion 以下 FA) が血管傷害にともなう様々な情報伝達に関与する可能性が指摘されている。今回 このような情報伝達に関わるとされる FA の構成分子のチロシンりん酸化が、バルーンカテーテルによる血管傷害によりどのように変化するか検討した。【方法】１２週令雄性 Sprague-Dawley ラットの両側総頸動脈および胸部大動脈を、sodium orthovanadate で前処置後摘出した。動脈中膜を界面活性剤を含む溶液中で細切・ホモゲナイズし得られた可溶画分を、抗りん酸化チロシン抗体で免疫沈降し、同抗体および種々の FA 構成蛋白に対する抗体で Western blot 分析を行った。また左頸動脈を Fogaty embolectomy catheter で傷害後、経時的に屠殺、同様の分析を行い、非傷害右頸動脈と比較した。【結果】正常大動脈および頸動脈では FA 構成蛋白である paxillin および focal adhesion kinase のチロシンりん酸化が著明であった。また talin、phosphatidylinositol 3-kinase の弱いチロシンりん酸化が認められた。バルーンカテーテル傷害後、focal adhesion kinase のチロシンりん酸化は著しく低下したが、paxillin では変化せず、phosphatidylinositol 3-kinase では増加した。傷害早期には PDGF レセプターおよび PDGF により活性化される phospho－lipase C-γ のりん酸化は認められなかった。【総括】バルーンカテーテル傷害により血管平滑筋と細胞外マトリックスとの接着部位に局在する情報伝達分子のチロシンりん酸化状態が変化した。この変化は増殖因子を介さず、カテーテルの機械的傷害により直接引き起こされると思われた。従来指摘されている増殖因子以外に機械的刺激が直接血管細胞の反応を惹起する可能性が示された。

Ⅳ. 助成金の使途内訳

助成金額 1,000,000 円

交通費 157,291 円　宿泊費 345,200 円　食　費 480,000 円

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Major tyrosine-phosphorylated proteins in the rat carotid artery, FAK and paxillin, are decreased after balloon injury.

血管傷害による血管壁内の細胞内情報伝達の変化。

ー特に接着斑のチロシンりん酸化蛋白の変化についてー

招聘責任者：　下門　顕太郎

国立循環器病センター研究所

招聘研究者：　宋　志清

泰安市中心医院　主治医師

Key words: cell adhesion molecules; muscle, smooth; remodeling; restenosis; balloon

Abstract

Background. Protein tyrosine phosphorylation plays an important role in signal transduction mediated by cytokines and vasoactive substances, and through the focal adhesion. However, the protein tyrosine phosphorylation in the normal and injured artery has not been elucidated. Therefore, we attempted to identify the major tyrosine-phosphorylated proteins in the normal artery, and their changes after balloon injury. **Methods and Results**. The normal and balloon-injured carotid arteries, the aorta, and other tissues from Sprague-Dawley rats were perfused with sodium vanadate, excised, and then homogenized in detergent-containing lysis buffer. The clarified tissue lysate was subjected to immunoprecipitation with anti-phosphotyrosine antibody, and then analyzed by Western blot with antibodies against focal adhesion kinase (FAK), paxillin, and phosphotyrosine. Western blot analysis with three different antibodies against phosphotyrosine revealed that pp68 and pp125 were the two major tyrosine-phosphorylated proteins present in the normal carotid artery and the aorta. Reprobing with various antibodies identified these proteins as paxillin and FAK. Immunodepletion with anti-phosphotyrosine removed FAK and paxillin, suggesting that most of these proteins were tyrosine-phosphorylated in the artery. The artery contained greater amount of tyrosine-phosphorylated FAK compared to the other tissues examined, including the inferior vena cava and the heart. Following the balloon injury of the carotid artery, but not after endothelial denudation ex vivo, the content of FAK and paxillin was decreased. **Conclusions** Our findings suggested that FAK and paxillin may play an important role in the maintenance of the normal structure and function, and in the response to balloon injury of the artery.

Tyrosine phosphorylation plays an important role in the signal transduction mediated by growth factors, cytokines, vasoactive substances, cell-cell or cell-matrix interaction, and mechanical stimuli (see reviews, 1 – 2). Based on studies with cultured vascular cells, tyrosine phosphorylation has been implicated in the maintenance of the structure and function of the normal artery[3 - 4] as well as in its responses to injury[5]. Activation of the PDGF receptor tyrosine kinase has been studied in the artery

in vivo [6]. However, the overall state of tyrosine phosphorylation in the normal and injured artery has not been elucidated.

Methods

Antibodies: Anti-phosphotyrosine (PY20), anti-focal adhesion kinase (FAK, clone 77), and anti-paxillin (clone 349) monoclonal antibodies were obtained from Transduction Laboratories (Lexington, Kentucky). Anti-phosphotyrosine (4G10) and anti-FAK (2A7) were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-phosphotyrosine rabbit IgG (RF-1) was a kind gift from Dr. Jiro Seki.

Tissue preparation: Eight-week-old male Sprague-Dawley rats were killed with a lethal dose of pentobarbital(50 mg/kg). After ice-cold sodium vanadate solution, containing 0.5mmol/L sodium ortho- vanadate, 10 mmol/L phosphate buffer(pH 7.4), 150 mmol/L sodium chloride, and 20 mmol/L hydrogen peroxide, was perfused through the left ventricle of the heart under pressure of 110 mm Hg, the carotid artery and the aorta were excised. After the adventitia was removed, the remaining arterial tissue was minced in cold RIPA buffer [7], and homogenized in a motor-driven Dawns homogenizer. Unsolubilized material was removed by centrifugation at 15,000 r.p.m. for 20 minutes at 4 ° C. The protein concentration of the resulting supernatant, used as tissue lysate, was determined by DC-protein assay kit (Bio-Rad, Hercules, CA). Each experiment was repeated at least three times with similar results.

Immunoprecipitation and Western blot analysis: Immunoprecipitation and Western blot analysis were conducted as reported previously [7], using RIPA buffer as the lysis buffer.

Immunodepletion: Since tyrosine phosphorylation of FAK and paxillin changes in response to various stimuli[8 – 14], we examined how much of FAK and paxillin is tyrosine-phosphorylated in the normal artery. Tyrosine-phosphorylated proteins in the tissue lysate were depleted by sequential immunoprecipitation with PY-20-Sepharose four times, and then FAK was immunoprecipitated with anti-FAK antibody.

Tissue distribution: For the study of the tissue distribution of FAK, tissue lysate was prepared from the heart, inferior vena cava, skeletal muscle, lung, kidney, and liver. Equal amount of protein (100 μg) from each tissue was used for immunoprecipitation with PY20.

Balloon injury: The left common carotid arteries of fifteen 10-week-old Sprague-Dawley rats were denuded of endothelium by three passages of a 2-F balloon catheter inserted through the left external carotid artery by a method reported previously[15]. After 24 h, 7 days and 17days, the content of tyrosine-phosphorylated proteins in the injured (left) and control (right) carotid artery was examined. Intraperitoneal administration of pentobarbital (30 mg /ml) and local administration of 1% xylocaine were used for anesthesia. All procedures were approved in advance by the institutional committee.

Ex vivo deendothelialization: To estimated the contribution of FAK and paxillin in the endothelium that was removed during the balloon injury, the endothelium of the aorta was removed ex vivo. The aorta was excised, transferred to cold sodium vanadate solution, and cut into two halves longitudinally. The endothelial cells of one half were removed with a surgical scalpel.

Results

Western blot analysis with three different anti-phosphotyrosine antibodies, PY20, 4G10 and RF-1, demonstrated that two major tyrosine-phosphorylated proteins, pp125 and pp68, were present in both the carotid artery and the aorta (data not shown). These bands were eliminated following addition of exogenous phosphotyrosine, but not following addition of phosphoserine or phosphothreonine, indicating that these bands represent specifically tyrosine-phosphorylated proteins. These two major tyrosine- phosphorylated proteins were identified as FAK and paxillin by probing the immunoprecipitate of tyrosine-phosphorylated proteins sequentially with PY20, anti-FAK, and anti-paxillin (Fig. 1, A). In a separate experiment where FAK and paxillin were immunoprecipitated with anti-FAK or anti-paxillin antibody, and probed with PY-20, both FAK and paxillin were also shown to be tyrosine phosphorylated (data not shown, but a similar finding also shown in Fig. 1, B, lane 8).

Following immunoprecipitation of tissue lysate depleted of tyrosine-phosphorylated proteins

with anti-FAK antibody, there was no FAK detected , while FAK was detected in the control lysate that was treated with protein G-Sepharose instead of PY20-Sepharose, indicating that most FAK was tyrosine-phosphorylated in the normal artery (Fig. 1, B). The same type of experiment conducted for paxillin demonstrated that all paxillin was tyrosine-phosphorylated in the normal artery (data not shown).

The aorta and the carotid artery contained more tyrosine-phosphorylated FAK than the inferior vena cava and the heart (Fig. 1C). The artery showed the greatest FAK content among the tissues tested, followed by the lung and the liver. As reported before[16], the kidney and the skeletal muscle had only small FAK content (Fig. 1C). Immunoprecipitation with anti-FAK antibody (data not shown) yielded the tissue distribution of FAK similar to those obtained by immunoprecipitation with PY20 (Fig. 1C).

Balloon injury of the carotid artery decreased the content of tyrosine-phosphorylated FAK and paxillin (Fig.2A). This decrease was detected as early as 24 h after the injury (Fig.2 B,C), through 17 days after the injury(Fig 2A, D), the last time point we examined. Immunoprecipitation with anti-FAK antibody revealed decrease of FAK protein (Fig 2 C) equivalent to the decrease of tyrosine-phosphorylated FAK detected by immunoprecipitation with anti-phosphotyrosine antibody (Fig 2 B), suggesting that the decrease was not due to the dephosphorylation of FAK or paxillin, but due to decrease of the content of these proteins themselves. The content of tyrosine-phosphorylated FAK and paxillin in the aorta deendothelialized ex vivo (Fig 2 E) was not different from that in the non-deendothelialized aorta, indicating that the decrease in FAK after the balloon injury was not due to loss of endothelial cells.

Discussion

In this study, we demonstrated that FAK and paxillin are two major tyrosine phosphorylated proteins in the normal rat artery, and characterized these proteins in the artery; most of these proteins are tyrosine phosphorylated in the normal artery; the artery contains larger amount of tyrosine-

phosphorylated FAK than the other components of the circulatory system; and balloon injury of the carotid artery decreases the content of these proteins. These findings suggest an important role of FAK and paxillin both in maintaining the normal structure and function of the artery, and in the development of vascular dysfunction after injury.

Most of the FAK and paxillin were tyrosine phosphorylated in the arterial wall as indicated by the failure to detect FAK or paxillin after immunodepletion of tyrosine-phosphorylated proteins. It is possible that FAK or paxillin are not tyrosine phosphorylated themselves, but are associated other tyrosine-phosphorylated proteins, and removed by immunoprecipitation with anti-phosphotyrosine. However, this is not likely because each of FAK and paxillin was tyrosine phosphorylated when it was immunoprecipitated with anti-FAK and anti-paxillin, respectively, and probed with anti-phosphotyrosine, and because the major tyrosine-phosphorylated protein was FAK or paxillin following the anti-FAK or anti-paxillin immunoprecipitation (data not shown).

It is interesting that the artery contained more FAK than the vein, while both consist a continuous circulatory system. This difference in FAK content may be partly explained by the difference in the blood pressures to which the artery and the vein are exposed, since the vein becomes arterialized when it is connected to the artery and exposed to a higher blood pressure [17]. This possibility is also supported by the observation that tyrosine phosphorylation of FAK in the heart is affected by the intraventricular pressure [14]. The difference of blood pressure may exert its effect directly through biomechanical sensors [18], indirectly through change in the matrix components[8 – 9], or through generation of molecules such as PDGF [10], insulin-like growth factor-I [11], thrombin [12], and angiotensin II [13]. Our finding that the artery is abundant in FAK accords with a previous report that the artery is a major tissue expressing FAK during the early development[16].

FAK and its tyrosine phosphorylation are implicated in the cardiac hypertrophy induced by pressure overload [14], in the sensing shear stress and subsequent increase of NO production by the endothelium [3], and in the survival of the cell [4]. Deficiency of FAK changes cell adhesiveness and cell motility, and causes lethal phenotype in FAK-knockout mice[19]. These reports suggest that the

tyrosine phosphorylation of these proteins plays an important role in signal transduction through the focal adhesion in the artery.

The vascular injury decreased the content of FAK and paxillin in the injured artery. This decrease was not solely due to the endothelial loss, since deendothelialization of excised aortas did not decrease the content of FAK. One potential mechanism of this decrease is that via growth factors generated at the injury sites. Insulin and insulin-like growth factor-I decrease tyrosine phosphorylation of FAK through insulin receptor in vitro [20]. However, there is no evidence that insulin receptor is activated in injured arteries. Most of other growth factors increase tyrosine phosphorylation of FAK [8 – 14]. More likely is that the mechanical stretch associated with the ballooning disturbed the interaction between smooth muscle integrins and the extracellular matrix, dephosphorylated FAK and paxillin, and subsequently decreased the content of these proteins. Detachment of cells from the matrix dephosphorylated FAK in vitro [21]. Based on the observation that balloon injury causes change of the matrix composition and smooth muscle integrins, it has been pointed out that signals transmitted through focal adhesion may play an important role in the development of restenosis after balloon angioplasty[22]. Our study provides new evidence that balloon injury impairs the intracellular signaling pathway of signals transmitted through the focal adhesion.

Acknowledgments

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Figure legends

Figure 1. Western blot analysis of tyrosine-phosphorylated proteins in arterial tissue lysates. **A:** Tissue lysate of the aorta (AO, lanes 1, 3, and 5) and the carotid artery (CA, lanes 2, 4, and 6) was immunoprecipitated with PY20, and then analyzed by Western blotting. Three antibodies, PY20 (lanes 1 and 2), anti-FAK (lanes 3 and 4), and anti-paxillin (lanes 5 and 6) were applied sequentially to the same nitrocellulose membrane. Arrows indicate positions of FAK and paxillin. Molecular sizes are indicated on the left. **B:** Tyrosine-phosphorylated proteins in tissue lysates of the aorta were depleted by repeated immunoprecipitation with PY20-Sepharose beads (lanes 1-3, and 9-11), and then FAK was immunoprecipitated with anti-FAK antibody (lanes 4 and 12). In control determinations, the tissue lysates were treated with protein-G Sepharose instead of PY20-Sepahrose (lanes 5-7, and 13-15), and then FAK was immunoprecipitated (lane 8, 16). The nitrocellulose membrane was probed sequentially with PY20 (lanes 1-8) and anti-FAK antibody (lanes 9-16). **C:** FAK was immunoprecipitated with PY20 from various tissues, and detected with anti-FAK antibody.

Figure 2. Effects of balloon injury. **A:** Seventeen days after deendothelialization of the left carotid artery with a balloon catheter, the right (cont) and left (injured) carotid arteries were excised, and tyrosine-phosphorylated proteins were analyzed as described in the legend to Fig. 1. The lysate was immunoprecipitated(IP) with PY20 and probed with the same antibody. Arrows indicate positions of FAK, paxillin and the antibody(IgG). Molecular sizes are indicated on the left. **B:** Twenty-four h after the balloon injury, FAK was immunoprecipitated with PY20, and probed with anti-FAK. **C:** Twenty-four h after the balloon injury, FAK was immunoprecipitated with anti-FAK, and probed with the same antibody. **D:** The same nitrocellulose membrane as in A was reprobed with anti-FAK and anti-paxillin antibodies. **E:** Ex vivo deendothelialization. The aorta was cut into two halves longitudinally. Endothelial cells of one half were removed ex vivo, and FAK was immunoprecipitated with PY20, and probed with anti-FAK.

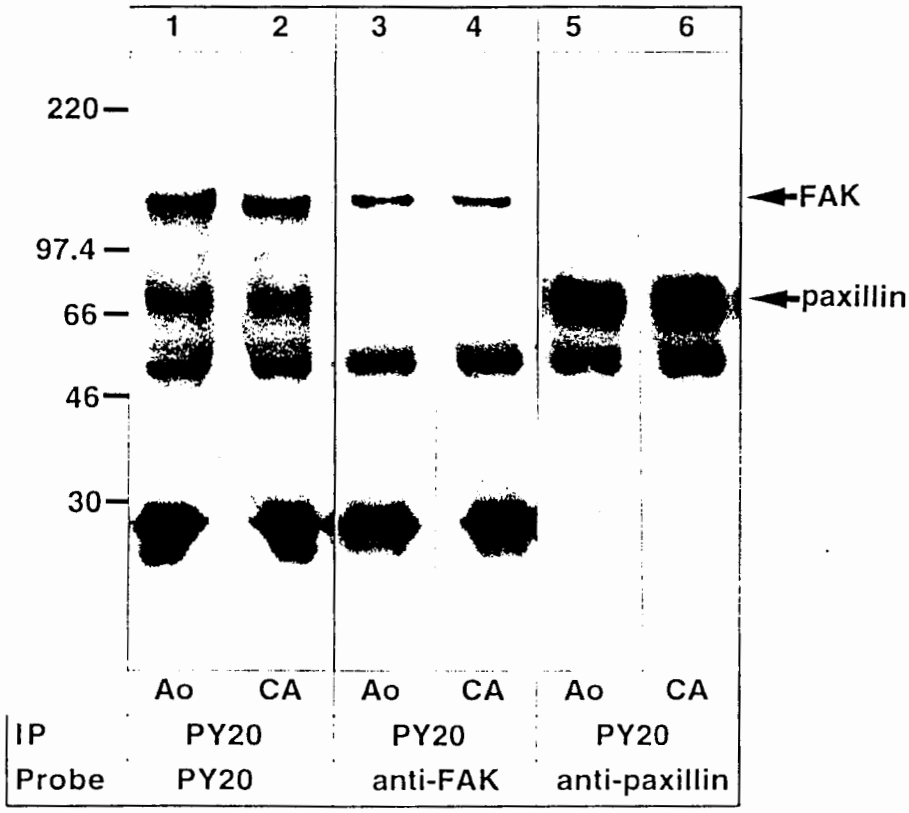
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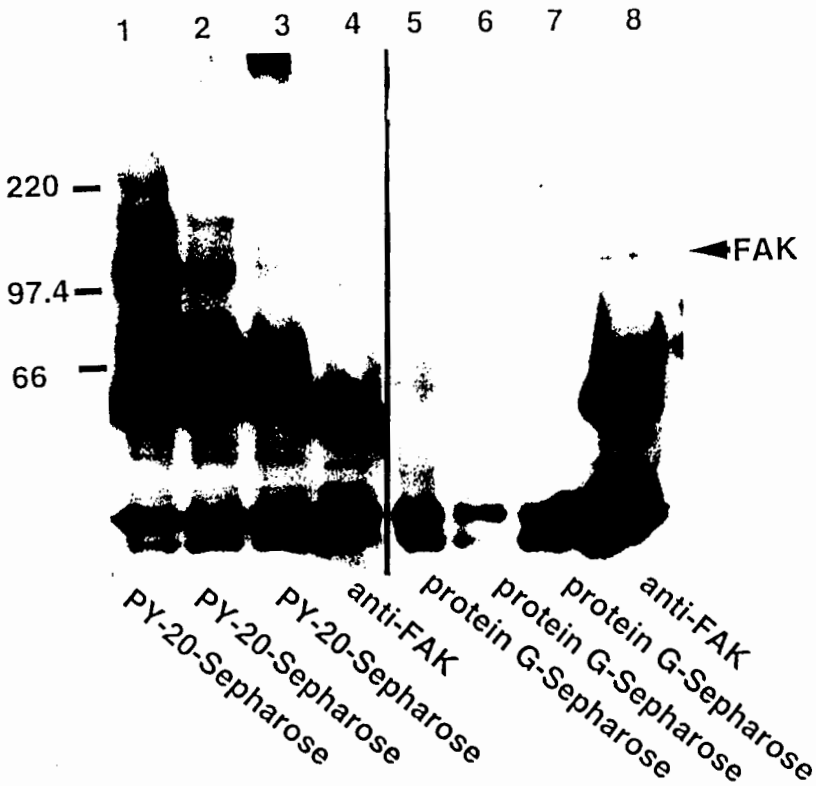
Fig 1A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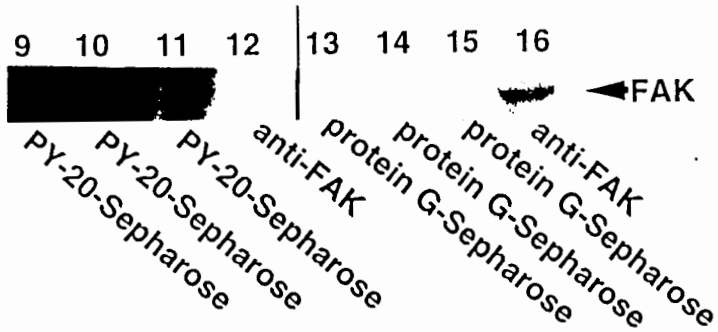
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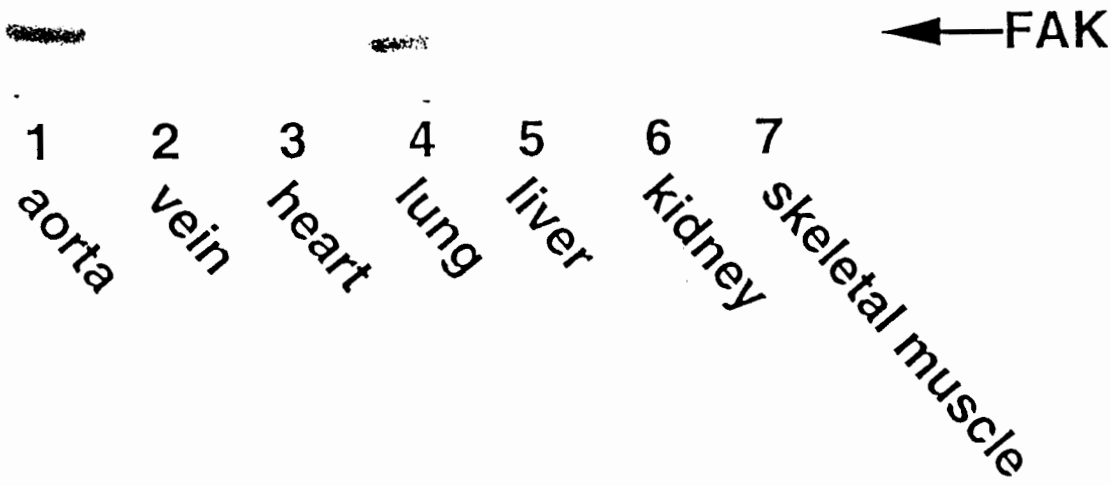
Fig 1B

Fig 1C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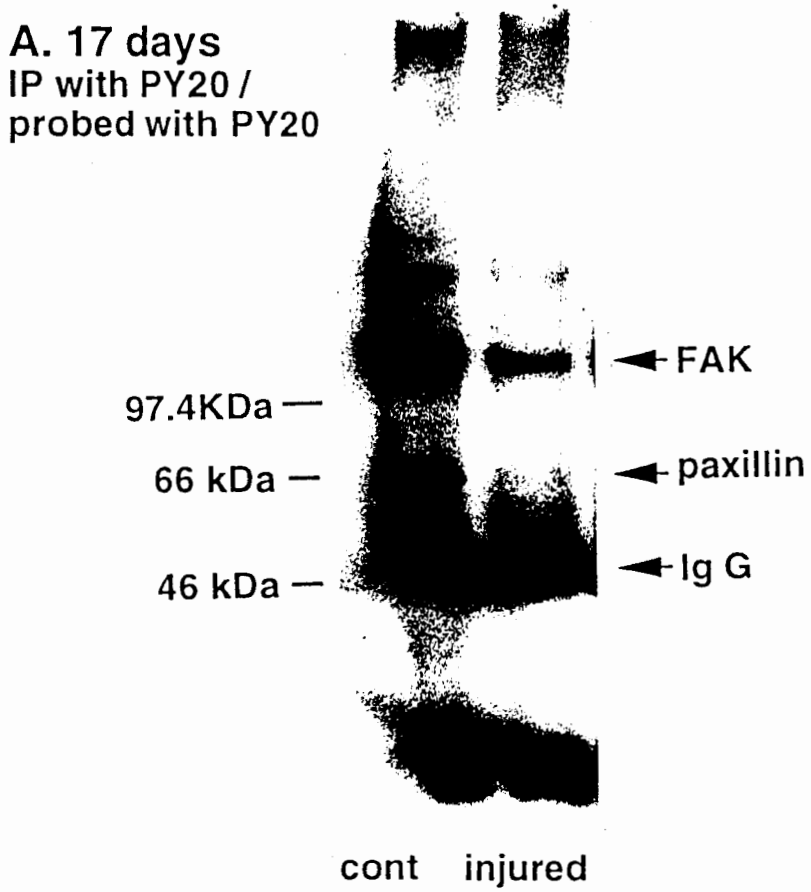

B. 24h. IP with PY20 / probed with anti-FAK

C. 24h. IP with anti-FAK / probed with anti-FAK

D. 17 days. IP with PY20 / probed with anti-FAK

probed with anti-paxillin

E. ex vivo deendothelialization. IP with PY20 / probed with anti-FAK

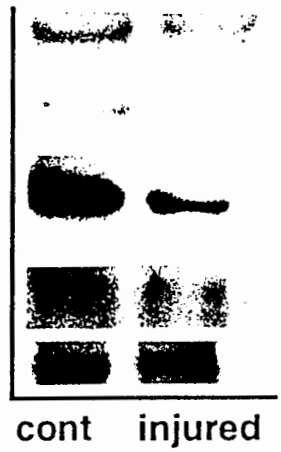

Fig 2

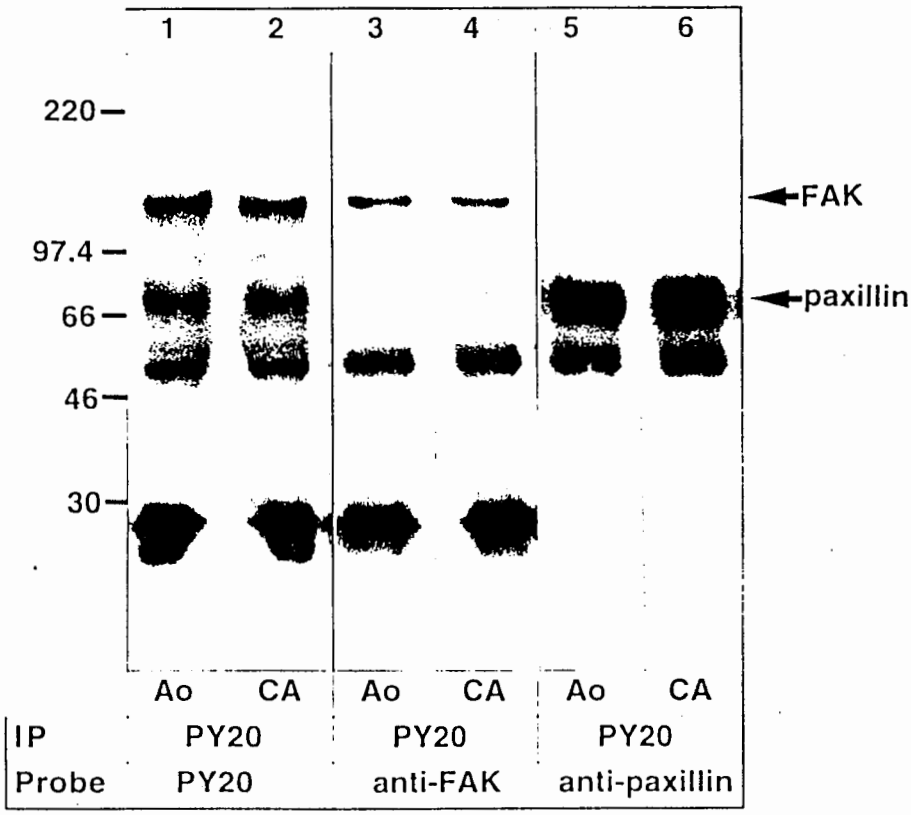
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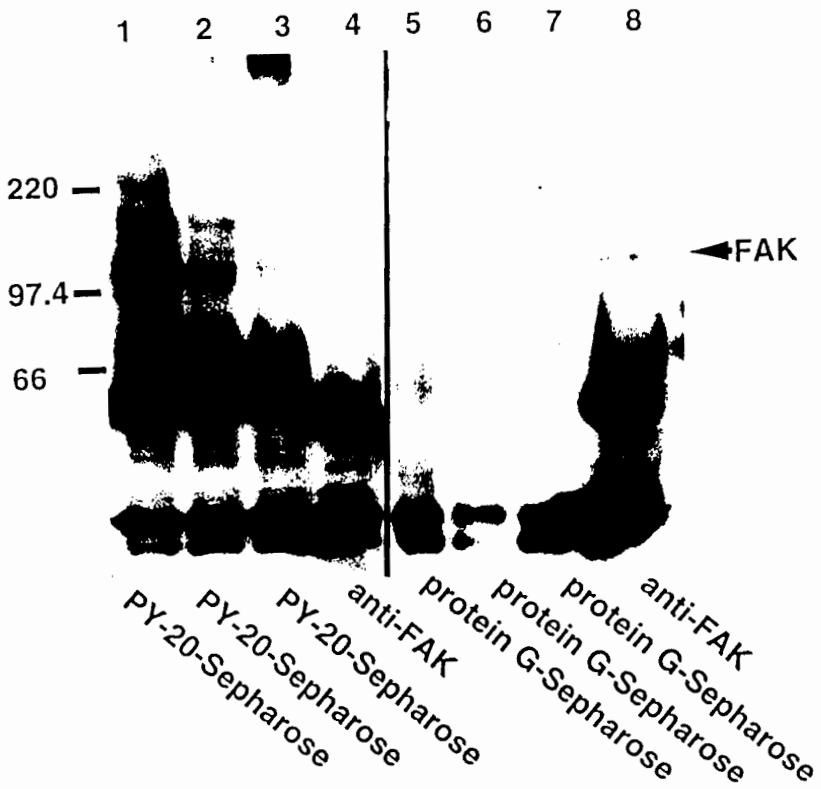
Fig 1A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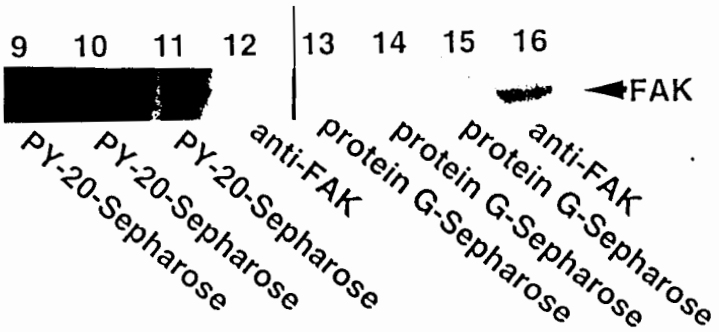
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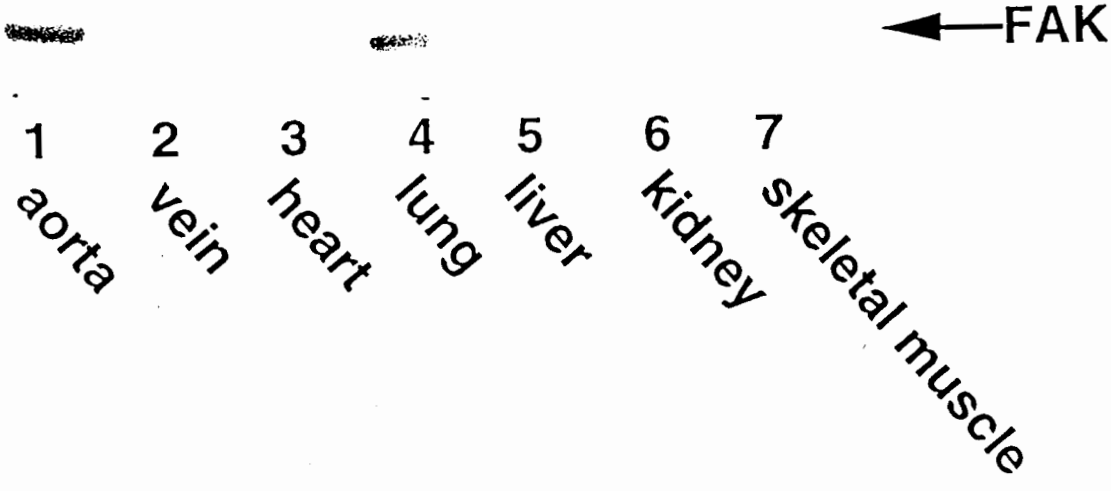
protein G-Sepharose
anti-FAK

Fig 1B

Fig 1C


FAK
1 aorta
2 vein
3 heart
4 lung
5 liver
6 kidney
7 skeletal muscle