PEDRO ALEXANDRE SIMÕES VENDEIRA

# MECANISMOS MODULADORES DA REGENERAÇÃO E DIFERENCIAÇÃO DA GLÂNDULA SUPRA-RENAL

## A esteroidogénese num modelo de autotransplante

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***Ao Professor***

***Doutor Manuel Miranda Magalhães***

***À Professora***

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***Ao meu Pai***

***À minha Mãe***

***À Lurdes***

***Ao André***

***Ao Bruno***

# PREFÁCIO

O despontar da minha actividade na área da investigação científica teve lugar em Fevereiro de 1987 quando, a convite da Professora Doutora Maria da Conceição Magalhães e do Professor Doutor Manuel Magalhães, iniciei a minha frequência no Instituto de Histologia e Embriologia da Faculdade de Medicina do Porto. Tomei conhecimento dos trabalhos de investigação em curso integrados no grupo de estudo da morfofisiologia da glândula supra-renal do rato, e iniciei a minha aprendizagem na área da microtomia, ultramicrotomia e morfologia ultrastrutural, tendo sido contratado como Monitor da disciplina de Biologia Celular e Molecular da Faculdade de Medicina do Porto em Outubro de 1988.

Na altura, as primeiras observações efectuadas pela Professora Doutora Maria da Conceição Magalhães em enxertos de glândula supra-renal sugeriram que os fenómenos de diferenciação adrenocortical e mecanismos moduladores envolventes poderiam ser estudados *in vivo* utilizando o procedimento do autotransplante, proporcionando um modelo de regulação não conseguido *in vitro* através da cultura de células.

Motivado por tais sugestões, desenvolvi o procedimento do autotransplante da glândula supra-renal do rato Wistar tendo introduzido alterações pessoais nas técnicas até então utilizadas, o que permitiu criar um modelo de estudo da regulação dos processos de regeneração e diferenciação a nível do tecido glandular adrenocortical.

O trabalho desenvolvido nesta dissertação encontra-se compilado em quatro publicações. A sua distribuição cronológica reflecte de uma forma coerente a evolução nas técnicas e conhecimentos adquiridos, e o seu conteúdo pretende proporcionar dados objectivos respeitantes às directrizes impostas. Um capítulo introdutório para enquadramento da dinâmica adrenocortical e dos

mecanismos de regeneração e diferenciação glandular afigura-se importante para uma leitura global esclarecedora e para uma melhor compreensão dos fenómenos envolvidos no autotransplante da glândula. As considerações finais inevitavelmente salientam os resultados já expostos nas publicações, mas constituem um meio necessário para uma mais profícua correlação dos mesmos, com vista a uma exposição mais clara das conclusões.

Os meus sinceros agradecimentos ao corpo docente, investigador e técnico do Instituto de Histologia e Embriologia, sem os quais nada teria sido possível.

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A ambos gostaria de expressar um sentimento. Na verdade, é algo mais que um sentimento, é a certeza de me terem transmitido que nada se cria sem o necessário empenho. Se esta dissertação viu a luz do dia, então eu sei a quem a devo, sei quem me ensinou a investigar e questionar, sei quem me orientou e motivou, sei a quem devo agradecer. É com toda a humildade que o faço. Pelo bem estar de todos nós, muito obrigado.

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Em obediência ao disposto no Decreto-Lei nº 388/70, Artigo 8º, parágrafo 2, esclareço que efectuei o planeamento e execução das experiências, observação do material e análise dos resultados e redigi as primeiras versões das seguintes publicações, que fazem parte integrante desta dissertação:

**I** **Vendeira, P., Magalhães, M. M. e Magalhães, M. C.**

Autotransplantation of the adrenal cortex: a morphological and autoradiographic study. *The Anatomical Record*, 232: 262-272, 1992.

**II** **Vendeira, P., Neves, D., Magalhães, M. M. e Magalhães, M. C.**

Modulation of autotransplanted adrenal gland by endothelin-1: a morphological and biochemical study. *The Anatomical Record*, 246: 98-106, 1996.

**III** **Vendeira, P., Pignatelli, D., Neves, D., Magalhães, M. M., Magalhães,M. C., Ho, M. e Vinson, G.**

New insights into zonal differentiation of adrenal autotransplants in the rat: an immunohistochemical study. *Journal of Endocrinology*, 149: 497-502, 1996.

**IV** **Vendeira, P., Pignatelli, D., Neves, D., Magalhães, M. M., Magalhães, M. C. e Vinson, G.**

Effects of prolonged infusion of basic fibroblast growth factor and IGF-I on adrenocortical differentiation in the autotransplanted adrenal: an immunohistochemical study. *Journal of Endocrinology*, 162: 21-29, 1999.

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# ÍNDICE

# INTRODUÇÃO

Durante os séculos da Renascença, após a descoberta das supra-renais em 1563 por Eustachii – *Glandulae renibus incumbentes* – estas glândulas foram consideradas como parentes embriológicos das gónadas, desprovidas de qualquer interesse médico. Em 1845 as regiões periférica e central do órgão foram designadas por Huschke respectivamente de córtex e medula. Dez anos mais tarde Bernard classifica-as como órgãos de secreção interna, e Addison salienta a sua importância na manutenção da vida – fez a primeira descrição clínica do deficiente funcionamento da glândula supra-renal e promoveu os primeiros estudos científicos sobre a fisiopatologia deste órgão. A evidência de que a exérese das glândulas supra-renais, em diversas espécies animais, tinha como consequência a morte (Brown-Séquard, 1856, 1857), alertou a comunidade científica para a necessidade da melhor compreensão da sua morfofisiologia.

Em 1866, estudos morfológicos de Arnold levaram este autor a considerar três zonas no córtex supra-renal, – glomerulosa, fasciculada e reticular –, denominação que se tem mantido até hoje, e que os estudos posteriores a nível de microscopia óptica e electrónica confirmaram. A demonstração de que a manutenção da vida é da responsabilidade do córtex da glândula supra-renal deve-se a Biedl (1913), Houssay e Lewis (1923) e Hartman e col. (1927), os quais realçam também a sua importância relativamente à zona medular. Esta zonação morfológica da glândula levou à exploração do conceito de anatomia funcional do órgão o que permitiu relacionar as características zonais do córtex supra-renal com a biossíntese dos esteróides, atribuindo-se a produção de mineralocorticóides à zona externa e a produção de glicocorticóides e androgénios à zona interna – zona fasciculada + zona reticular – (Neville e O'Hare, 1979).

Décadas de investigação e consequentes descobertas no campo da morfofisiologia da esteroidogénese adrenocortical permitiram verificar que muitos dos conceitos adquiridos apresentam importância fundamental na compreensão e no significado funcional da glândula. No entanto, é curioso notar como a nível dos conhecimentos actuais de Biologia Celular e Molecular, notamos grandes dificuldades em responder com exactidão às mesmas perguntas colocadas pelos primeiros investigadores desta área. Como regenera a glândula supra-renal? Como se diferencia?

## 1. A esteroidogénese na glândula supra-renal

O conhecimento do mecanismo da esteroidogénese na glândula supra-renal desenvolveu-se a partir dos anos 30, após o isolamento químico e identificação de numerosos princípios activos esteróides do córtex supra-renal. Os trabalhos pioneiros de Kendall (1937) e Pfiffner (1942), constituiram a base científica do início da corticoterapia, verificando-se que a administração de extractos lipídicos adrenocorticais permitiam um adequado controle metabólico em animais supra-renalectomizados bilateralmente (Swingle e Pfiffner, 1930).

Todas as hormonas adrenocorticais conhecidas são, de facto, esteróides e a sua biossíntese implica a formação do denominado núcleo esteróide (Hayano e col., 1956). A demonstração de que a glândula supra-renal sintetiza colesterol e, a partir deste, as hormonas esteróides, deve-se a Hechter e col. (1953), que introduziram, pela primeira vez, o conceito do núcleo esteróide (o anel ciclopentanoperidrofenantreno). Este precursor pode ser sintetizado na glândula a partir do acetato, mobilizado a partir de colesterídeos de localização intracelular (gotículas lipídicas), ou ser obtido a partir da circulação sanguínea (Werbin e Chaikoff, 1961; Brown e col., 1979), sendo esta última a via preponderante através da endocitose de lipoproteínas (Gwynne e Strauss, 1982).

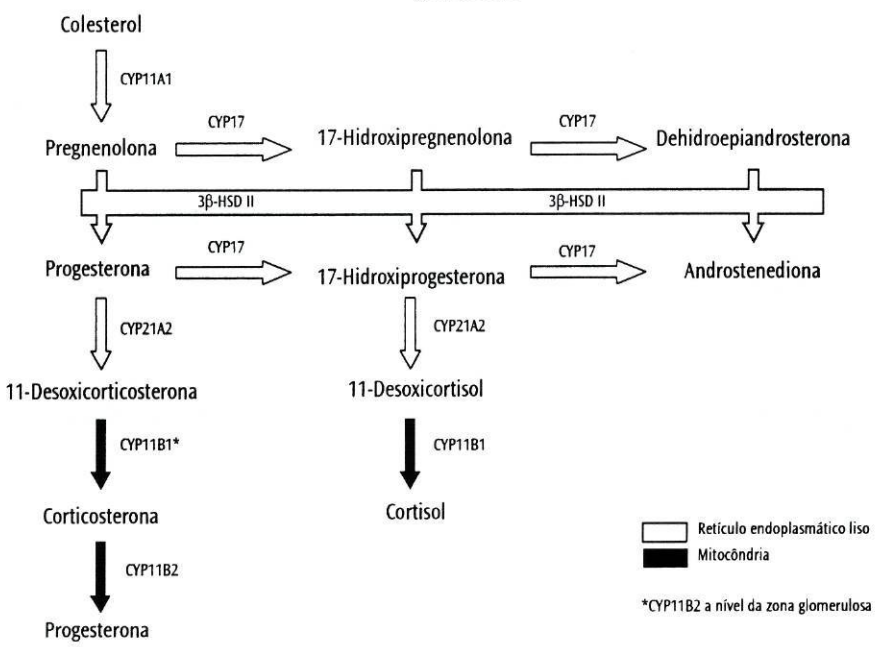
Os processos metabólicos necessários à actividade de biossíntese dos corticosteróides estão essencialmente dependentes da acção da família de enzimas esteroidogénicas CYP (citocromo P450) (Simpson e col., 1969; Miller, 1988; Nonaka e col., 1991; Fardella e Miller, 1996). Estas enzimas transferem electrões do NADPH para o oxigénio molecular com concomitante oxidação de diversos substractos. Dada a sua diferente localização subcelular, a actividade enzimática da esteroido-

génese distribui-se em diversos compartimentos, a que correspondem diferentes fases da biossíntese dos esteróides.

O passo limitativo de toda a "cascata" esteroidogénica tem como base o transporte do colesterol livre através do citoplasma até à membrana mitocondrial interna, local de actuação da CYP11A1 (enzima de clivagem da cadeia lateral do colesterol; $P450_{scc}$), promovendo a conversão do colesterol em pregnenolona (Mitani e col., 1982; Waterman e Simpson, 1985; Farkash e col., 1986). Neste processo deve salientar-se a proteína StAR (steroid acute regulatory protein), cuja acção na regulação aguda da esteroidogénese foi proposta em 1994 por Clark e col. Esta proteína é induzida pela acção da hormona adrenocorticotrófica (ACTH) e do monofosfato de adenosina cíclico (AMPc), sendo responsável pelo aumento do transporte de colesterol para a membrana mitocondrial interna (Stocco e Clark, 1996a,b; Stocco, 1999; Roy e col., 2000).

No quadro seguinte podem ser visualizados os principais passos da biossíntese dos esteróides no córtex supra-renal. Pode observar-se ainda a divisão das vias de síntese mineralocorticóide, glicocorticóide e androgénica, bem como os respectivos precursores e a localização enzimática subcelular.

***QUADRO***




(Quadro adaptado de Orth e Kovacs, 1998)

A pregnenolona pode posteriormente ser convertida em progesterona por intermédio da enzima 3β-HSD II (desidrogénase 3β-hidroxiesteróide/isomérase-$\Delta^{4,5}$; 3β-HSD), uma das raras enzimas envolvidas na esteroidogénese que não faz parte da família dos CYP (Rheaume e col., 1991). No entanto, nas espécies que expressam a enzima CYP17 (17α-hidroxílase/17,20-líase; $P450_{C17}$), a pregnenolona pode ser convertida em 17-hidroxipregnenolona (Bradshaw e col., 1987), o que permite, por um lado, a sua manutenção como precursor glicocorticóide e, por outro, a possibilidade da biossíntese de androgénios. Da mesma forma, na presença de CYP17, a progesterona pode ainda ser convertida em 17-hidroxiprogesterona, mantendo as mesmas vias de conversão esteróide. A presença de CYP17 é exclusiva da zona interna da glândula supra-renal nas espécies que a expressam (Miller, 1988, 1999).

Em alguns mamíferos, nomeadamente no rato, a existência de CYP17 é posta em dúvida (Simpson e Waterman, 1988). Desta forma, a via preferencial de biossíntese, quer dos glico, quer dos mineralocorticóides, passa pela conversão de progesterona em 11-desoxicorticosterona catalizada pela enzima CYP21A2 (21-hidroxílase; $P450_{C21}$). Na presença de CYP17, como ocorre na espécie humana, a via glicocorticóide preferencial consiste na conversão de 17-hidroxiprogesterona em 11-desoxicortisol catalizada pela CYP21A2 (Kominami e col., 1980), o que condiciona a conversão de progesterona em 11-desoxicorticosterona como passo quase específico da via mineralocorticóide.

Na via glicocorticóide no córtex supra-renal a conversão final consiste na hidroxilação em C-11 de 11-desoxicortisol, catalizada pela enzima CYP11B1 (11β-hidroxílase; $P450_{C11}$), originando o cortisol como glicocorticóide predominante no ser humano (Mitani e col., 1982; Chua e col., 1987; Simpson e Waterman, 1988). No caso do rato, por provável ausência de CYP17, o glicocorticóide predominante é a corticosterona (Bush, 1953; Magalhães e col., 1974), obtida pela conversão de 11--desoxicorticosterona em presença da enzima CYP11B1. Estas conversões são observadas sobretudo a nível da zona fasciculada e em menor grau na zona reticular.

A principal hormona mineralocorticóide no homem e no rato é a aldosterona, e no que diz respeito aos passos finais da via mineralocorticóide, a conversão fundamental nos seres humanos, e

no rato, consiste na hidroxilação em C-11, mas tendo como substracto a 11-desoxicorticosterona, obtendo-se a corticosterona. Este passo é catalizado pela CYP11B2 (síntase da aldosterona; $P450_{C11AS}$). Esta enzima substitui a CYP11B1 a nível da zona glomerulosa no que diz respeito às reacções de hidroxilação, e preside também à conversão de corticosterona em aldosterona (Lauber e col., 1987; Ogishima e col., 1989; Imai e col., 1990; Curnow e col., 1991; Müller, 1991; Müller e col.. 1991; Vinson e col., 1991, 1992), conversão esta verificada apenas nesta zona (Giroud e col., 1956; Vinson e col., 1995).

Em relação à síntese de androgénios, a presença de CYP17 é a condição fundamental para a obtenção de dehidroepiandrosterona (DHEA) e androstenediona. Estes esteróides de baixa actividade androgénica, bem como a forma sulfatada da DHEA, são os mais específicos da glândula supra-renal e sintetizados sobretudo a nível da zona reticular (Hornsby, 1985).

A maior parte das enzimas envolvidas na esteroidogénese estão localizadas na fracção microssómica, a qual, nas células secretoras de esteróides, é composta sobretudo por retículo endoplasmático liso (Hall, 1984; Ishimura e Fujita, 1997). No entanto, as enzimas que promovem a clivagem da cadeia lateral do colesterol (CYP11A1) bem como a hidroxilação em C-11 (CYP11B1), estão localizadas na membrana mitocondrial interna. A conversão de 11-desoxicorticosterona em aldosterona é catalizada por uma única enzima também de localização mitocondrial (CYP11B2), incluindo as hidroxilações em C-11 e C-18 e a metil oxidação em C-18, sendo estas últimas reacções exclusivas da zona glomerulosa.

Desta forma, na glândula supra-renal, um precursor esteroidogénico é transportado entre diversos compartimentos intracelulares até à obtenção do composto final (Nussdorfer, 1986). As sequências enzimáticas, nomeadamente nas conversões finais das vias glico e mineralocorticóide, diferem na sua distribuição cortical, obedecendo a aspectos fundamentais e específicos da regulação zonal que permanecem ainda hoje pouco clarificados.

Os mecanismos de regulação do transporte intracelular e da secreção de hormonas esteróides carecem ainda de uma maior compreensão. Ao contrário da tese inicial de que o armazenamento de hormonas esteróides se processava a nível das gotículas lipídicas (Rhodin, 1971), novas linhas de evidência sugerem que estas hormonas não são armazenadas, nem libertadas de forma intermitente após estimulação (Nussdorfer, 1980, 1986; Black, 1992). Desta forma, a sua síntese e secreção é provavelmente contínua, ocorrendo a regulação ao nível da síntese (ciclo secretor constitutivo). Aproximadamente 100 compostos esteróides diferentes podem ser extraídos das glândulas humanas e de animais experimentais, mas apenas alguns são habitualmente segregados, exercendo actividade biológica significativa (Black, 1992).

# 2. Dinâmica adrenocortical

## *2.1. Conceitos morfofisiológicos*

No seu conjunto, as glândulas supra-renais constituem um dos principais reguladores homeostáticos do organismo.

A presença íntima de dois componentes tecidulares endócrinos, com origem embriológica e tipo de secreção diferentes, tem vindo a ganhar progressiva importância na definição dos mecanismos morfofisiológicos, constituindo actualmente um modelo de estudo da regulação hormonal parácrina, dada a evidência que córtex e medula estão não só morfológica mas também funcionalmente interligados (Carballeira e Fishman, 1980; Nussdorfer, 1986; Hinson, 1990; Andreis e col., 1992; Bornstein e col., 1994; Einer-Jensen e Carter, 1995; Ehrhart-Bornstein e col., 1996; Mazzocchi e col., 1998; Pignatelli e col., 1998a; Vinson e Ho, 1998; Bornstein e Vaudry, 1998; Nussdorfer e Mazzocchi, 1998).

De facto, estudos embriológicos demonstraram que o córtex supra-renal tem origem nas células do mesênquima adjacentes à cavidade celómica e que se encontram em íntima proximidade com a crista urogenital (Uotila, 1940; Gruenwald, 1946). Esta glândula supra-renal fetal é posteriormente invadida por células neuroectodérmicas provenientes da crista neural e que formarão a medula supra-renal (Le Douarin, 1980; Rovasio e Thiery, 1987). Esta proximidade anatómica é ainda veiculada pelos sistemas vascular e nervoso que proporcionam um elo de conexão entre os dois componentes da glândula. No que diz respeito ao primeiro, o córtex é profusamente irrigado, observando-se um exuberante plexo vascular localizado na região subcapsular que origina as artérias corticais e medulares (Lever, 1952; Rhodin, 1971). As artérias corticais sofrem divisão já a

nível do córtex, promovendo a sua irrigação, e os seus ramos terminais podem ser observados na região medular. As artérias medulares cruzam o córtex mas apenas se dividem em ramos medulares (Gagnon, 1957). Desta forma, e ao contrário do córtex, a medula exibe um duplo aporte vascular, permitindo a obtenção de elevadas concentrações locais de corticosteróides, com efeito estimulador inequívoco a nível da síntese de adrenalina (Wurtman e Axelrod, 1966; Pohrecky e Rust, 1968; Sparrow e Coupland, 1987).

A inervação principal da glândula depende de fibras simpáticas pré-ganglionares. Estas fibras atravessam o córtex estabelecendo sinapses com as células cromafins da medula responsáveis pela síntese e secreção de catecolaminas, e que assim se comportam como fibras pós-ganglionares (Côrte-Real, 1948; Coupland, 1965a,b, 1989; Douglas, 1966; Schramm e col., 1975; Unsicker e col., 1989). A maior parte da inervação destinada ao córtex foi interpretada inicialmente como exclusiva dos vasos sanguíneos (Kleitman e Holzwarth, 1985; Engeland e col., 1985), desempenhando um papel na regulação do fluxo sanguíneo glandular. Existe, no entanto, evidência da presença de terminações nervosas e mesmo de células cromafins em íntima relação com grupos dispersos de células glandulares adrenocorticais (Côrte-Real, 1948; Holzwarth e col., 1987; Gallo-Payet e col., 1987; Bornstein e col. 1990, 1994; Charlton, 1990; Carlsson e col., 1990; Black, 1992; Bornstein e Ehrhart-Bornstein, 1992; Gilchrist e col., 1993), cujo significado será posteriormente discutido.

A divisão morfológica cortical em zona glomerulosa, fasciculada e reticular (Arnold, 1866) constituiu a base dos estudos referentes à compreensão da fisiologia adrenocortical. Numerosos investigadores prosseguiram estes trabalhos sobre a estrutura e a ultrastrutura da glândula (Kolmer, 1918; Greep e Deane, 1949a; Sabatini e Robertis, 1961; Greep, 1961; Deane, 1962; Long e Jones, 1967; Idelman, 1970; Rhodin, 1971; Motta, 1979; Neville e O'Hare, 1982a; Nussdorfer, 1986; Ogishima e col., 1992). Salientam-se alguns investigadores portugueses no desenvolvimento dos estudos da histofisiologia supra-renal (Celestino da Costa, 1911, 1951; Côrte-Real, 1945, 1949;

Vasconcelos Frazão, 1954) e, posteriormente, nas investigações da estrutura fina do córtex supra-renal (Magalhães, 1972, 1974, 1976; Magalhães e Magalhães, 1980).

A zona glomerulosa ocupa a porção mais periférica da glândula totalmente rodeada pela cápsula fibrosa que a envolve, seguida da zona interna (zona fasciculada + zona reticular) que constitui a maior parte do órgão. Esta última rodeia a medula supra-renal que assim constitui o núcleo da glândula.

Em estudos recentes assiste-se frequentemente à discussão do significado da zona intermédia adrenocortical (Neville e O'Hare, 1982a; Ganguly, 1991; Mitani e col., 1994, 1999; Miyamoto e col., 1999). Trata-se de uma camada de células glandulares descrita em 1937 por Hall e Korenchevsky, e denominada de zona de transição por Deane e Greep (1946). O termo zona intermédia deve-se a Cater e Lever (1954), dada a sua localização entre a zona glomerulosa e fasciculada, não havendo, no entanto, evidência inequívoca que sugira a sua função como elemento de transição zonal. Este facto, aliado à sua ausência em diversas espécies torna o seu papel funcional ainda hoje de muito difícil interpretação.

Nos estudos em microscopia óptica (Neville e O'Hare, 1982a; Nussdorfer, 1986) as células da **zona glomerulosa** formam pequenos ninhos glandulares esféricos localizados por baixo da cápsula. As suas dimensões são inferiores às da zona interna, apresentam uma relação citoplasma/núcleo menor, um número reduzido de inclusões lipídicas e núcleos de menores dimensões, com maior abundância de heterocromatina. A **zona fasciculada** é constituída por cordões paralelos de células glandulares separados por delicadas trabéculas fibrovasculares. É característica a presença de citoplasma vacuolar devido à grande abundância de inclusões lipídicas. A **zona reticular** demarca-se da fasciculada pela presença de cordões anastomosantes de configuração irregular separados por capilares sinusoidais. O citoplasma é escasso em gotículas lipídicas e contém numerosos grânulos de lipofuscina. As células da **zona intermédia**, quando identificáveis, são caracterizadas por uma depleção completa de inclusões lipídicas, cujo significado será posteriormente discutido. Com o advento dos estudos ultrastruturais foi possível definir determinadas características das

células glandulares corticais, permitindo a sua colocação no grupo das células secretoras de esteróides (Lever, 1955; Sabatini e Robertis, 1961; Nishikawa e col., 1963; Sheridan e Belt, 1964; Magalhães, 1974). Assim, as observações em microscopia electrónica revelaram células da zona glomerulosa exibindo mitocôndrias de pequenas dimensões, alongadas e com cristas lamelares e/ou tubulares, perfis de retículo endoplasmático liso em quantidade moderada e ocasionais gotículas lipídicas. Ao contrário, as células da zona fasciculada apresentam mitocôndrias de forma esférica ou ovóide com cristas tipicamente vesiculares, numerosos perfis de retículo endoplasmático liso com arranjo vesicular em "favo de colmeia" e abundantes inclusões lipídicas. A zona reticular é fundamentalmente caracterizada por mitocôndrias atípicas com cristas mistas tubulo--vesiculares, retículo endoplasmático liso muito compactado e grande abundância de grânulos de lipofuscina. As células da zona intermédia apresentam mitocôndrias semelhantes às da zona reticular, sendo a sua principal característica ultrastrutural a total ausência de inclusões lipídicas, confirmando as observações efectuadas em microscopia óptica.

A actividade secretora do córtex é fundamentalmente controlada pelo eixo hipotálamo-hipofisário (Taylor e Fishman, 1988) e pelo sistema renina-angiotensina (Quinn e Williams, 1988). As hormonas esteróides produzidas interferem nas múltiplas respostas do organismo ao stress, em estreita relação com os mecanismos de homeostasia (Axelrod e Reisine, 1984; Munck e col., 1984). As suas acções estão fundamentalmente implicadas no metabolismo glicídico e proteico (glicocorticóides), bem como no equilíbrio hidroelectrolítico e regulação da pressão arterial (mineralocorticóides). A medula está sujeita ao controle do sistema nervoso autónomo libertando catecolaminas (Ungar e Phillips, 1983), responsáveis por variadas funções homeostáticas e de resposta ao stress, incluindo a actividade rítmica cardíaca e a actividade do músculo liso visceral e dos vasos sanguíneos (Hoffman e Singer, 1967; Vaughan e Blumenfeld, 1998).

A hormona adrenocorticotrófica, sintetizada e segregada pelo lobo anterior da hipófise (Sayers, 1950), é um peptídeo de 39 aminoácidos responsável pela estimulação da secreção de glicocorti-

cóides, esteróides androgénicos e, em menor quantidade, de mineralocorticóides pelo córtex supra-renal (Hechter, 1949; Tucci e col., 1967; Nussdorfer e col., 1978; Nussdorfer, 1980, 1986; Tait e col., 1987; Cozza e col., 1989; Biglieri, 1989). A sua secreção é fundamentalmente controlada pela presença de hormona libertadora de corticotrofina (CRH), proveniente de neurónios hipotalâmicos e veiculada pelo sistema portal hipotálamo-hipófise (Saffran e col., 1955; Hermus e col., 1984; Antoni, 1986; Jones e Gillham, 1988). A libertação deste peptídeo obedece a eferências cerebrais, tendo sido já demonstrada a sua dependência de mecanismos de "feed-back" negativo veiculados pelos glicocorticóides (Itoi e col., 1987; Dallman e col., 1987; Beyer e col., 1988). A dependência da síntese e libertação da ACTH deste mecanismo de "feed-back" é um fenómeno bem conhecido (Ingle e col., 1938). A ligação da ACTH a receptores glandulares corticais activa a adenilcíclase desencadeando um aumento dos níveis de AMP cíclico que por sua vez levam à activação da proteína-cínase dele dependente (proteína-cínase A) e determina a fosforilação de várias proteínas (Gill, 1976; Fujita e col., 1979; Kurscheid-Reich e col., 1992; Cone e Mountjoy, 1993; Penhoat e col., 1993). A principal acção da ACTH é o incremento da síntese e secreção do cortisol no homem e da corticosterona no rato por acção a nível da zona fasciculada. Quer no respeitante à ACTH, quer às hormonas esteróides, existe um ritmo circadiano no homem, caracterizado sobretudo por concentrações elevadas destas no início do período de despertar, atingindo os valores mais reduzidos no início do período de sono. No rato, este ritmo é invertido, dada a presença de actividade nocturna com alteração dos padrões de sono (Krieger, 1977). A acumulação de glicocorticóides na glândula é mínimo bem como o seu conteúdo em colesterol, o que se correlaciona com a presença de actividade contínua de síntese de esteróides (Dickerman e col., 1984; Hall, 1985). Os efeitos da ACTH podem ser do tipo agudo e crónico consoante ocorrem em alguns minutos ou apenas após algumas horas ou dias (Simpson e Waterman, 1983). O efeito agudo da ACTH implica um aumento da conversão de colesterol em pregnenolona a que não é alheia a acção da proteína StAR (Clark e col., 1994; Peters e col., 1998; Stocco, 1999), mecanismo este independente da transcrição genética (Lin e col., 1995; Waterman, 1995; Kim e col., 1997).

Um efeito precoce da ACTH é o aumento do fluxo sanguíneo glandular (Maier e Staehelin, 1968). Ao contrário, os efeitos crónicos da ACTH envolvem um aumento da síntese da maior parte das enzimas esteroidogénicas e das endomembranas intimamente relacionadas, bem como um efeito global a nível da síntese de ácidos nucleicos e crescimento da glândula sobretudo à custa da zona fasciculada (Grower e Bransome, 1970; Nussdorfer e col., 1978; DuBois e col., 1981; Nussdorfer e Mazzocchi, 1983; Simpson e Waterman, 1988; Chouinard e Fevold, 1990; Boshier e col., 1990; Provencher e col., 1992; Perry e col., 1992; Ehrhart-Bornstein e col., 1998). A este respeito, concentrações suprafisiológicas de ACTH induzem hiperplasia e hipertrofia adrenocortical e a deficiência em ACTH induz a atrofia da glândula (Gill, 1972; Nickerson, 1975; Parker e col., 1983). De acordo com Dallman (1984), a hiperplasia é um fenómeno tardio, ao contrário da hipertrofia cuja evidência constitui prova inequívoca da capacidade trófica desta hormona sobre o córtex supra-renal.

A aldosterona, o mais potente mineralocorticóide circulante é produzido exclusivamente nas células glandulares glomerulosas (Miao e Black, 1982; Crivello e col., 1983). A sua secreção é regulada fundamentalmente pela angiotensina II e potássio (Laragh e col., 1960; Davis e col., 1963; Nussdorfer, 1980; Quinn e Williams, 1988), sendo a ACTH, como já referido, um modulador de menor importância. Tal como na regulação pela ACTH, a acção do potássio implica um aumento dos níveis intracelulares de AMP cíclico (Kojima e col., 1985). No entanto, o seu principal mecanismo de acção é exercido através da despolarização da membrana citoplasmática, com activação de canais de cálcio, permitindo o aumento do afluxo intracelular deste (Kramer e col., 1980; Kojima e col., 1985). Todos estes factores actuam por regulação a nível da conversão do colesterol em pregnenolona e ainda na conversão de corticosterona em aldosterona catalisada por uma única enzima mitocondrial (CYP11B2), como referimos (McKenna e col., 1978; Aguilera e Katt, 1978, 1979; Quinn e Williams, 1988). A angiotensina II, principal efector do sistema renina-angiotensina, é produzida a nível pulmonar por acção da enzima de conversão da angiotensina

cujo substrato é a angiotensina I. Esta é produzida a partir do angiotensinogénio hepático, por acção da enzima renina, sintetizada a nível das células justaglomerulares renais (Gibbons e col., 1984). É efectivamente a este nível, que a sua regulação é definida pela pressão arterial renal e ainda pela concentração de sódio a nível do tubo contornado distal detectado pela mácula densa (Speckart e col., 1977).

Ao contrário do mecanismo observado com a ACTH, a angiotensina II não actua promovendo o aumento dos níveis de AMP cíclico (Fujita e col., 1979). Após a ligação a receptores da superfície celular de alta afinidade (Peach e Dostal, 1990; Rainey e col., 1992a), este peptídeo promove a activação da fosfolípase C, levando ao aumento dos níveis de inositol trifosfatado (IP3), com posterior aumento da concentração de cálcio livre intracelular (Capponi e col., 1994; Vinson e col., 1994a).

A restrição de sódio na dieta bem como a administração crónica de angiotensina II provocam hipertrofia da zona glomerulosa e aumentam a secreção de aldosterona, sem interferir com a zona fasciculada (Deane e col., 1948; Giacomelli e col., 1965; Kenyon e col., 1978; Nussdorfer, 1980; Riondel e col., 1987; McEwan e col., 1995; Pignatelli e col., 1998b). Os mecanismos ligados à mitogénese induzida pela angiotensina II permanecem ainda pouco claros. Para além do aumento da expressão de CYP11B2 na zona glomerulosa acompanhando o aumento da esteroidogénese mineralocorticóide (Shibata e col., 1991, 1993), Ogishima e col. (1992) sugerem um efeito proliferativo deste peptídeo sobre as células glomerulosas dada a expressão aumentada da enzima CYP11B2, sem no entanto demonstrarem claramente a expressão celular mitótica (Mitani e col., 1994).

## *2.2 O modelo do autotransplante*

Numerosos modelos experimentais têm vindo a ser utilizados na tentativa de promover a regeneração e diferenciação da glândula supra-renal *in vivo*.

De acordo com os mecanismos atrás descritos, e da necessidade de um melhor esclarecimento da morfofisiologia adrenocortical, o autotransplante da glândula supra-renal constitui um modelo de grande utilidade no estudo da regeneração da célula glandular (Ingle e Higgins, 1938a; Skelton, 1959; Saxe e Connors, 1985; Taki e Nickerson, 1985; Belloni e col., 1990; Vendeira e col., 1992, 1996), permitindo a reprodução parcial de uma zonação morfológica adrenocortical com capacidade funcional.

Para além do intuito puramente experimental com vista à compreensão destes mecanismos, numerosos investigadores procuraram aplicar os métodos de autotransplante ao homem, com vista à correcção de determinadas situações de hipocorticalismo. Desta forma, procurou-se ultrapassar as situações de terapêutica esteróide crónica (bem como as suas consequências a longo prazo), e prevenir o aparecimento do síndrome de Nelson, passível de afectar até cerca de 40% dos indivíduos submetidos a supra-renalectomia bilateral. Promover o crescimento *in vivo* de tecido adrenocortical funcionante por autotransplante, previamente vascularizado ou não, seria uma solução prática, tecnicamente exequível e aparentemente viável. As situações mais frequentes, com possível aplicação desta técnica, dizem respeito à necessidade de efectuar a supra-renalectomia bilateral devido às recidivas da microcirurgia hipofisária transesfenoidal por doença de Cushing, síndrome de produção ectópica de ACTH, raras situações de síndrome de Cushing por hiperplasia macronodular, e feocromocitoma bilateral associado ou não a síndromes de neoplasia endócrina múltipla tipo II.

Após revisão da literatura foi possível encontrar os resultados de 115 doentes submetidos a autotransplante de glândula supra-renal (Ibbertson e O´Brien, 1962; Bricaire e Philbert, 1965; Ledingham e col., 1966; Drucker e col., 1967; Bayer e col., 1971; Zieleniewski e Stapor, 1972; Kaplan e Shires, 1972; Oliveira e col., 1976; Hardy, 1978; Prinz e col., 1979; Barzilai e col., 1980; Klempa e col., 1980; Urban e col., 1980; Belli e col., 1984; Ott e col., 1984; Hardy e col., 1985; Xu e col., 1989, 1992; Matsuda, 1987; Demeter e col., 1990; Dickstein e col., 1991; Miao e col., 1991;

Lucon e col., 1993; Erdogan e col., 1994; Okamoto e col., 1996; Miyauchi e col., 1999), tendo o primeiro enxerto não vascularizado sido efectuado por Franksson e col. (1959). Dentro do grupo dos enxertos não vascularizados (73), o local de eleição para o autotransplante foi o tecido muscular. Seguramente, pela manutenção do território vascular, a taxa de sucesso foi francamente melhor no grupo dos enxertos vascularizados onde se obteve uma melhor percentagem de resultados moderados e bons (26 em 42), ao contrário da obtida no primeiro grupo, isto é, enxertos não vascularizados (24 em 73). Esta comparação de resultados deve-se à definição bioquímica da necessidade de corticoterapia substitutiva, suplemento da mesma em situações de stress, e autonomia completa comprovada pela resposta glicocorticóide à administração de ACTH.

Evidentemente, a utilização de enxertos vascularizados implica anastomoses microvasculares, apenas passíveis de serem efectuadas em circunstâncias muito particulares, o que não é compatível com uma utilização alargada. Resta pois a outra possibilidade, isto é, a utilização de enxertos não vascularizados; no entanto, a viabilidade dum enxerto deste tipo permanece muito pouco atractiva, situação essa que tem incrementado a necessidade de uma investigação mais profunda no que diz respeito aos mecanismos de regeneração e diferenciação do tecido glandular cortical.

O estudo em animal experimental é basilar, dada a facilidade de execução e a simplicidade técnica do procedimento, juntamente com a possibilidade (vedada no homem), de prescindir de corticoterapia de substituição sem que daí advenha a morte do animal (Saxe e Connors, 1985; Vendeira e col., 1992; Sarría e col., 1995). A preservação da secreção endógena de glico e mineralocorticóides, por intermédio de autotransplante, seria ideal sempre que as circunstâncias o exigissem, razão pela qual a pesquisa neste campo se tem arrastado desde o século XIX, tendo o primeiro autotransplante no rato sido efectuado com sucesso em 1898 por Poll (citado por Wyman e Suden, 1932).

Numerosos locais de autotransplante, e diversos animais experimentais, têm sido investigados ao longo das décadas, procurando explorar factores locais de facilidade regeneradora contribuindo

para a taxa de sucesso. Como citado por Penney e col. (1963), através de uma excelente revisão, os locais de autotransplante variam de forma heterogénea desde a tiróide, peritoneu, útero, testículo, fígado, rim, ouvido, cérebro e até a câmara anterior do olho. Curiosamente, grande parte dos autores considera a colocação intramuscular o local de eleição (Jaffe e Plavska, 1926; Penney, 1963; Saxe e Connors, 1985; Belloni e col., 1990). Este facto deve-se, por um lado, à facilidade da intervenção, dada a acessibilidade do local de implantação, e por outro lado, à profusa rede vascular do tecido muscular, facilitando a revascularização dos enxertos e sua consequente regeneração e diferenciação. A este respeito, é de referir que o conceito de regeneração completa é discutível. No procedimento de enucleação, caracterizado por extrusão do conteúdo glandular *in situ*, e constituindo um dos modelos de regeneração adrenocortical (Ingle e Higgins, 1938b; Greep e Deane, 1949b; Macchi e Wyman, 1960), considera-se a regeneração completa ao fim de 30 dias. Da mesma forma, e em estudos imunohistoquímicos com marcadores específicos de zona, observa-se uma zonação funcional adrenocortical ao fim deste período de tempo após o processo de enucleação (Engeland e Levay-Young, 1999; Ulrich-Lai e Engeland, 2000). Em localização intramuscular esta evidência é adquirida apenas aos 60 dias (Penney e col., 1963), 90 dias (Wiman e Suden, 1932), ou até posteriormente, acima dos 120 dias (Belloni e col., 1990, 1991), o que revela a falta de critérios definitivos de regeneração. A relativa precocidade da regeneração cortical após enucleação é seguramente atribuída à manutenção da circulação sanguínea, local de regeneração *in situ* e ao mínimo traumatismo produzido durante o procedimento.

Como já previamente referido, as técnicas de autotransplante da glândula supra-renal, e nomeadamente as de localização intramuscular agradam pela simplicidade, rapidez de execução e alta taxa de sucesso dada a enorme capacidade regenerativa do tecido adrenocortical no rato (Geiringer, 1954; Penney e col., 1963; Belloni e col., 1991). A análise dos resultados, também em função do tipo de enxerto utilizado, permitiram verificar que a viabilidade celular e a capacidade regenerativa do enxerto estão relacionadas com a quantidade de tecido capsular e de tecido parenquimatoso

implantados (Srougi e Gittes, 1978; Saxe e Connors, 1985). Estas observações são resultado de trabalhos experimentais prévios que utilizaram grande quantidade de tecido glandular adrenocortical no implante (Penney e col., 1960; Penney, 1963; Belloni e col., 1990), o que permitiu verificar nas fases precoces pós-autotransplante (2º e 3º dias), a presença de um vasto infiltrado de tipo inflamatório com grande abundância de células macrofágicas envolvendo o tecido cortical que se apresenta na sua quase totalidade necrosado, com excepção de um pequeno grupo de células glandulares de localização subcapsular. Penney e col. (1960) referem ainda que nesta fase de regeneração, o tecido viável constitui cerca de 3 a 4% do volume total do autotransplante, e sugerem em função de estudos morfológicos em microscopia electrónica centrados no tipo de mitocôndrias e retículo endoplasmático liso, bem como no número de gotículas lipídicas (Penney, 1963), que a função primordial das células corticais nos primeiros dias do autotransplante está centrada na proliferação e síntese proteica, enquanto o substrato morfológico da esteroidogénese apenas está definido a partir da primeira semana. De acordo com Brooks (1961), a fragmentação múltipla do tecido enxertado constitui um factor importante no autotransplante de tecido endócrino, e de facto, Belloni e col. (1990) verificaram que a necrose cortical e a presença do infiltrado inflamatório podem ser ultrapassados pelo implante preferencial de um maior número de fragmentos contendo pequenas quantidades de tecido capsular (e células glandulares subcapsulares anexas) em detrimento do tecido cortical, permitindo provavelmente uma facilitação dos processos de neovascularização e consequente nutrição precoce do enxerto.

O autotransplante de glândula supra-renal apresenta-se como uma massa de tecido adrenocortical regenerado. Tal como inicialmente descrito (Jaffe e Plavska, 1926; Wiman e Suden, 1932; Ingle e Higgins, 1938a), o tecido medular não regenera, observando-se por vezes, a presença de tecido cicatricial preenchendo a região central da glândula. Todo o córtex entra em necrose após o procedimento, com excepção da cápsula e de pequenos ninhos celulares subcapsulares (Brenner e col., 1953; Belloni e col., 1982), a que se segue um fenómeno de restauração tecidular denomi-

nado regeneração adrenocortical (De Groot e Fortier, 1959; Skelton, 1959; Srougi e col., 1980), sem evidência de restauração medular, provavelmente pela alta sensibilidade destas células à anóxia, perda de inervação autónoma ou dificuldade de acesso à neovascularização. Existem, no entanto, algumas referências com descrição de células cromafins após autotransplante da glândula (Wyman, 1928; Turner, 1939; Coupland, 1957, 1958).

A possibilidade de células conjuntivas capsulares contribuirem para a regeneração cortical por diferenciação glandular foi inicialmente proposta por Zwemer e col. (1938) e reafirmado por Baxter (1946) e Butcher (1948). Este conceito foi posteriormente abandonado a partir dos estudos de Greep e Deane (1949b) que não observaram conversão glandular das células capsulares. Este mesmo resultado foi posteriormente confirmado por Brenner e col. (1953), e desde então têm vindo a ser consideradas como células estaminais, no processo de regeneração cortical, as células glandulares subcapsulares. Este conceito não afasta obviamente a possibilidade da existência de mecanismos reguladores locais com proveniência capsular, nomeadamente na influência da polaridade zonal glandular, como sugerido recentemente por Vinson e Ho (1998). Independentemente do seu papel específico, a implantação de tecido glandular desprovido de cápsula resulta na ausência de regeneração do enxerto, salientando o importante papel da mesma, ou então das células glandulares que lhe estão aderentes no momento do autotransplante (Ingle e Higgins, 1938a; Dempster, 1955; Saxe e Connors, 1985; Vendeira e col., 1992).

Observações sobre a regeneração de tecido cortical demonstraram a presença de um volume glandular idêntico ao do tecido enxertado, o que permitiu concluir que, morfologicamente, a regeneração estaria provavelmente concluída entre o primeiro e segundo mês (Geiringer, 1954). Srougi e Gittes (1978), embora reafirmando este período como o tempo de regeneração cortical máxima, descrevem a massa tecidular total obtida como inferior à inicialmente implantada, e de facto, também em estudos utilizando o modelo da enucleação, Pellegrino e Torcigliani (1957) e De Groot e Fortier (1959) referem que o volume total de tecido regenerado não ultrapassa 60% do volume da glândula intacta. Da mesma forma, Belloni e col. (1990) referem a presença de nódu-

los de tecido adrenocortical regenerado no fim do primeiro mês, embora a massa tecidular não seja adequada para restaurar as concentrações plasmáticas normais de corticosterona.

É de salientar, no entanto, que a forma de avaliar o sucesso do autotransplante varia com o tempo em função da modificação e desenvolvimento de novas técnicas analíticas. Enquanto Ingle e Higgins (1938a) apenas utilizam a morfologia de luz, Skelton (1959) acrescenta o doseamento de corticosterona, colesterol e ácido ascórbico na glândula e no plasma. Gibson e Krieger (1981), Engeland (1984, 1986) e Okamoto e col. (1992), procedem ao estudo do ritmo da corticosterona plasmática em condições basais e de stress. Sem dúvida, a microscopia electrónica permitiu enriquecer o estudo das alterações morfológicas observadas nos enxertos adrenocorticais. Desde os trabalhos pioneiros de Penney (1963) e Penney e col. (1963), numerosos autores procuraram desenvolver o estudo da morfologia fina dos enxertos em regeneração, no intuito de elucidar alterações estruturais, como possível base de formação de teorias funcionais (Belloni e col., 1990; Vendeira e col., 1992). Seki e col. (1969), avaliando a regeneração após enucleação bilateral, em função de parâmetros bioquímicos e morfológicos (microscopia electrónica e autorradiografia em microscopia de luz), sugerem uma rápida proliferação de células adrenocorticais (dados autorradiográficos utilizando $^{3}$H-timidina) nos dias iniciais pós-autotransplante e que estaria praticamente concluída no primeiro mês.

Presentemente, os doseamentos plasmáticos de ACTH, corticosterona e aldosterona, bem como a actividade da renina constituem pilares fundamentais na avaliação bioquímica do enxerto ao longo do tempo, avaliando a sua capacidade funcional. Da mesma forma, e para além dos estudos morfológicos, também a utilização de métodos imunohistoquímicos específicos, capazes de delimitar grupos funcionais adrenocorticais (Laird e col., 1988; Ho e col., 1994; Pignatelli e col., 1995) e, assim, possibilitar a identificação dos tipos celulares envolvidos no processo de regeneração e diferenciação pós-autotransplante, constituem elementos importantes destes estudos.

De uma forma geral mas ainda não absoluta, a maior parte dos autores referem que mesmo após

o período em que a regeneração é dada como concluída (o que não é consensual), os padrões de funcionalidade do autotransplante não atingem os do animal intacto. Esta afirmação é válida no que diz respeito à produção e secreção de corticosterona. No entanto, a análise global dos resultados revela que, em muito maior escala, é o mecanismo da esteroidogénese dos mineralocorticóides, que se encontra francamente deficitário a avaliar pelas concentrações de aldosterona plasmática (Ulrich-Lai e Engeland, 2000).

Estudos morfológicos e funcionais suportam a existência de uma interacção parácrina entre córtex e medula supra-renal, dada a proximidade anatómica e a co-localização de células corticais e medulares (Bornstein e col., 1992, 1994, 1997).

A este respeito, e dada a identificação de numerosos peptídeos reguladores identificados na medula supra-renal, e com acção na célula adrenocortical (Hinson, 1990; Malendowicz, 1993; Hinson e col., 1994a,b), é atractivo pensar que a ausência de regeneração medular poderá ser parcialmente responsável pela deficiente regulação parácrina a nível da zona glomerulosa subcapsular, condicionando os seus mecanismos de diferenciação e regulação esteroidogénica. Já a nível da zona fasciculada, a acção da ACTH poderá compensar a deficiente regulação medular, tal como observado pelo aumento significativo das concentrações de corticosterona após administração deste peptídeo (Rebuffat e col., 1991), não se observando a mesma situação no que diz respeito à aldosterona. De acordo com o princípio de Halsted (1909), a actividade de um tecido endócrino necessita de um ambiente sistémico favorável; na sua ausência, isto é, numa situação de défice hormonal, desencadeiam-se mecanismos que levarão à estimulação do crescimento do tecido endócrino. Estes dados são apoiados por Wyman e Suden (1937), utilizando como tecido endócrino o córtex supra-renal autotransplantado, e, de facto, a administração de ACTH parece facilitar a regeneração enquanto a administração de corticosteróides no pós-operatório a inibe (Srougi e col., 1980), sendo este facto apontado como uma das principais causas da falência do autotransplante humano (Hardy, 1978), onde não é possível privar o indivíduo de doses substitutivas de corticosteróides, impedindo o aparecimento de concentrações suprafisiológicas de ACTH.

## *2.3 Mecanismos da regeneração e diferenciação glandulares*

O conceito de que o córtex supra-renal é responsável pela síntese e secreção de hormonas esteróides distintas, de acordo com a zona considerada, permitiu levantar questões relevantes no que diz respeito aos factores que regulam esta subespecialização bioquímica. De facto, a expressão única de determinadas enzimas por zona cortical, a especificidade destas enzimas para diferentes intermediários na "cascata" esteroidogénica e a sua diferente organização subcelular a nível das membranas dos organelos, constituem factores passíveis de desempenhar um papel relevante quer a nível qualitativo, quer a nível das proporções absolutas e relativas dos esteróides sintetizados.
A este propósito não é alheio o facto do arranjo das zonas corticais em camadas concêntricas, conferir características funcionais particulares à dinâmica glandular, complementadas por uma relação específica com os eixos vasculares e inervação existentes. Da mesma forma, alterações morfofuncionais na distribuição zonal do córtex adquirem grande importância na compreensão dos mecanismos de regulação endócrina e parácrina, fundamentais no desempenho da resposta esteroidogénica.

Os mecanismos de proliferação, regeneração e diferenciação adrenocorticais estão ainda hoje pouco clarificados, nomeadamente na glândula adulta, onde a fisiologia da manutenção do volume cortical permanece por esclarecer na totalidade. Durante o desenvolvimento embrionário e pós-natal, a maioria das divisões celulares ocorre no córtex supra-renal periférico, nomeadamente nas zonas glomerulosa e fasciculada externa (Ford e Young, 1963; Wright, 1971; Dhom, 1973; Belloni e col., 1978), ou na zona definitiva do córtex fetal (Johannisson, 1979). Após a obtenção da maturação cortical, a taxa de divisão celular diminui de forma a permitir um fenómeno homeostático capaz de manter um balanço com a morte celular existente. Desta forma, torna-se claro que o desenvolvimento da glândula supra-renal bem como a zonação cortical daí decorrente, exigem um balanço dinâmico entre crescimento, diferenciação funcional e morte celular fisiológica; aparentemente, os fenómenos de proliferação celular localizam-se nas regiões mais exter-

nas da glândula, enquanto a morte celular seria uma característica predominantemente observada nas regiões mais internas, nomeadamente na região justamedular (Wyllie e col., 1973; Zajicek e col., 1986; Schwartzman e Cidlowski, 1993; Mitani e col., 1998). Curiosamente, o ritmo de proliferação celular na zona glomerulosa parece exceder o necessário à sua manutenção e, ao contrário, este mesmo ritmo parece ser insuficiente a nível da zona reticular (Wright, 1981), corroborando observações prévias de que na regulação do volume adrenocortical da glândula adulta, a maior parte dos fenómenos proliferativos é também da responsabilidade do córtex externo (Wright, 1971; Wright e col., 1973; Payet e col., 1980). Tendo em conta estes dados, uma das hipóteses mais vulgarmente aceites, no que diz respeito à manutenção do volume e fisiologia corticais, poderá envolver mecanismos preferenciais de divisão celular a nível da zona glomerulosa, migração centrípeta, diferenciação em tipos glandulares da zona fasciculada e finalmente senescência e morte na zona reticular (Deane, 1962; Ford e Young, 1963; Nussdorfer, 1980; Bertholet, 1981; Kataoka e col., 1996; Morley e col., 1996; Wolkersdörfer e col., 1996; Wolkersdörfer and Bornstein, 1998). De facto, células em estado de degeneração não estão confirmadas na zona glomerulosa, ao contrário da zona reticular onde Kerr e col. (1972) e Wyllie e col. (1973) descreveram um mecanismo de morte celular denominado de apoptose.

No entanto, e a este propósito, a problemática da zonação adrenocortical continua em aberto, sendo presentemente aceites três teorias básicas na sua génese e manutenção: migração celular, zonal e transformação de campo (Nussdorfer, 1980, 1986). Uma extensão da teoria zonal denomina-se teoria da proliferação intermédia e visa clarificar o papel da zona intermédia nos mecanismos de renovação celular (Idelman, 1978). A existência de numerosos argumentos contraditórios obriga a que nenhuma das teorias seja universalmente aceite.

A teoria mais facilmente compreensível, à luz do conhecimento actual em morfofisiologia e regulação endócrina supra-renal, será a teoria zonal (Swann, 1940). Esta, na sua essência, defen-

de que a zona glomerulosa é independente do controle pituitário, segregando mineralocorticóides, enquanto a zona interna depende da ACTH pituitária para a secreção de glicocorticóides (Chester Jones, 1948). A proliferação celular ocorre em todas as zonas corticais (Sarason, 1997), tal como sugerido pela observação de estudos autorradiográficos com $^{3}$H-timidina (Walker e Rennels, 1961; Hunt e Hunt, 1964; Nussdorfer, 1986). No entanto, esta teoria é fortemente contestada por outros autores, cujas observações sugerem que os mecanismos de zonação não obrigam necessariamente à existência prévia de 3 zonas distintas, defendendo os fenómenos migratórios como explicação mais coerente. Assim, a teoria da migração celular proposta por Gottschau em 1883, e posteriormente desenvolvida por Zwemer e col. (1938) e Celestino da Costa (1951), exprime o conceito de que as células glandulares de novo originam-se na periferia do córtex, migram centripetamente e degeneram na fronteira entre a zona reticular e a medula, local por vezes denominado de zona de senescência celular (Kerr e col., 1972; Wyllie e col., 1973; Almeida e col., 1998). Um dos principais argumentos a favor desta teoria é a explicação da regeneração cortical após enucleação (Ingle e Higgins, 1938b; Skelton, 1959; Mitani e col., 1995; Engeland e col., 1995), autotransplante (Ingle e Higgins, 1938a; Belloni e col., 1990; Vendeira e col., 1992) ou crescimento e diferenciação de células adrenocorticais em cultura (Turley, 1980; Roskelley e Auersperg, 1993). De facto, na ausência de fenómenos inequívocos que demonstrem a migração celular na glândula supra-renal intacta, é de salientar que a observação dos fenómenos proliferativos nas situações apontadas torna claro que a regeneração e diferenciação do córtex são de responsabilidade capsular (Zwemer e col., 1938; Baxter, 1946; Gruenwald, 1946; Williams, 1947) ou mais provavelmente das células glomerulosas subcapsulares (Greep e Deane, 1949b; Chester Jones e Spalding, 1954; Nickerson e col., 1969). De acordo com Côrte-Real (1949), baseado num vasto estudo sobre a actividade mitótica no tecido capsular, a responsabilidade deste tecido é de grande importância na proliferação adrenocortical. No entanto, e de acordo com os dados de Uotila (1940), não se observam transformações a nível dos fibroblastos capsulares, e daí a possibilidade admitida por estes autores de que no decorrer do desenvolvimento embrionário, algumas células do esboço

cortical seriam retidas pelos elementos mesenquimatosos da futura cápsula e aí se manteriam durante a vida, conservando as potencialidades de diferenciação e proliferação peculiares às células embrionárias.

A teoria da transformação de campo apoia-se no conceito de que a zona fasciculada é a região activamente secretora da glândula, enquanto as zonas glomerulosa e reticular constituem áreas de reserva de células (campos de transformação externo e interno, respectivamente), podendo, mediante os estímulos adequados entre os quais se salienta a acção da ACTH, serem transformadas em células fasciculadas (Tonutti, 1951; Chester Jones, 1957).

A denominada zona intermédia tem recentemente sido apontada como um dos prováveis pontos de localização de células progenitoras glandulares, reforçando a base da teoria da proliferação intermédia como variante da teoria da migração celular, na tentativa de clarificar os mecanismos de zonação adrenocortical (Idelman, 1978; Mitani e col., 1999). De acordo com esta teoria, células provenientes desta zona migrariam para a zona glomerulosa sendo a posterior migração efectuada para as zonas internas, mecanismo este dependente da ACTH (Golder e Boyns, 1973; Belloni e col., 1978). Esta zona, tal como recentemente foi demonstrado por técnicas imunohistoquímicas (Mitani e col., 1999), contém células desprovidas de CYP11B1 e CYP11B2, estando pois impossibilitadas de proceder à síntese de cortisol ou aldosterona, mas exibindo uma incorporação preferencial de bromodesoxiuridina detectada em estudo prévio por métodos imunohistoquímicos (Mitani e col., 1994). Estes dados apontam para uma localização preferencial das células progenitoras nesta zona ou na sua directa proximidade. Persistem dúvidas sobre se os fenómenos proliferativos relacionados com esta camada celular, estarão na origem de células com características morfofisiológicas da zona glomerulosa e/ou da zona fasciculada, uma vez que ambas as situações são despoletadas respectivamente pela estimulação da glândula com angiotensina-II e ACTH, levando a um aumento da expressão de CYP11B2 e CYP11B1 no tecido cortical (Ogishima e col., 1992; Mitani e col., 1996).

Independentemente dos fenómenos proliferativos se localizarem nas zonas glomerulosa ou intermédia, estudos autorradiográficos utilizando timidina tritiada sugerem um movimento centrípeto das células (Zajicek e col., 1986). Além disso, a administração crónica de ACTH modifica o fenótipo das células glomerulosas e intermédias para o tipo fasciculada (Kahri, 1968; Hornsby e col., 1974; Neville e O'Hare, 1982b; Gomez-Sanchez, 1985; Hornsby, 1985, 1987), estabelecendo um importante conceito dinâmico de zonação, ao considerar a interconversão de tipos celulares zonais distintos.

Com base nestas observações, existe a possibilidade de células progenitoras específicas para cada tipo celular ou de uma célula progenitora única, poderem veicular a resposta proliferativa a nível do córtex supra-renal. Desta forma, quer a capacidade de diferenciação da célula progenitora, quer a manutenção do volume adrenocortical deverão estar relacionados com a presença de factores endócrinos, nervosos e locais actuando de forma parácrina.

A hormona adrenocorticotrófica e a angiotensina II parecem estar prioritariamente relacionadas com esta manutenção (Liddle e col., 1954), actuando como morfogénios endócrinos e parácrinos essenciais, e condicionando a sua acção à amplificação produzida por factores de crescimento e de transcrição relacionados com os mecanismos subcelulares da esteroidogénese adrenocortical (Hornsby e col., 1983; Vane e col., 1990; Feige e Baird, 1991; Luo e col., 1994; Vinson e Ho, 1998). Outros factores de actuação local, com possível efeito morfogénico, incluem neuropeptídeos, nomeadamente o neuropeptídeo Y, a substância P e o peptídeo intestinal vasoactivo (VIP), dada a demonstração da sua localização nas regiões externas da glândula onde exercem efeito mitótico e esteroidogénico (Mazzocchi e Nussdorfer, 1987; Hinson e col., 1994a,b; Bornstein e col., 1994; Vinson e col., 1994b).

Ainda, os factores de origem vascular, nomeadamente a endotelina-1 poderão estar envolvidos. De facto, este peptídeo tem revelado funções importantes a nível da diferenciação celular, actu-

ando como sinal parácrino em diversos sistemas biológicos (Lerman e col., 1990; Chabrier e Braquet, 1990; Simonson e Dunn, 1991; Doherty, 1992; Battistini e col., 1993; Sakurai e Goto, 1993; Takuwa, 1993; Simonson, 1993; Masaki, 1993; Lotersztajn, 1993), incluindo um claro efeito estimulador na proliferação e capacidade esteroidogénica da zona glomerulosa (Mazzocchi e col., 1990a,b; Cozza e Gomez-Sanchez, 1990; Hinson e col., 1991; Mazzocchi e col., 1992; Belloni e col., 1996; Nussdorfer e col., 1997).

Apesar de pouco clarificado em termos de proliferação celular, a angiotensina II estimula a hipertrofia da zona glomerulosa, aumentando a secreção de aldosterona, sem interferir com o volume ou função da zona fasciculada (Riondel e col., 1987; Tian e col., 1995; Breidert e col., 1996; McEwan e col., 1999). Para além deste aspecto, a estimulação de células fetais de supra-renal com este peptídeo induz a diferenciação de células fenotipicamente glomerulosas (Rainey e col., 1992b).

A hormona adrenocorticotrófica é, sem dúvida o principal factor endócrino regulador da actividade proliferativa e secretora do córtex supra-renal. No entanto, a sua acção desenvolve-se prioritariamente por mecanismos de hipertrofia glandular a nível das zonas internas (Nickerson, 1975; Ehrhart-Bornstein e col., 1998). O seu efeito hiperplásico é tardio (Imrie e col., 1965), e de facto, trabalhos experimentais em culturas de células sugerem que este peptídeo não é um factor mitogénico directo para as células adrenocorticais (O'Hare e Neville, 1973; Hornsby e Gill, 1978; Rainey e col., 1983). A sua actividade mitogénica indirecta é provavelmente fruto da acção combinada com outros factores de produção local (Feige e Baird, 1991). De acordo com Ho e Vinson (1995), esta acção é amplificada pela actuação de factores de crescimento nomeadamente o IGF-I e FGF-2.

O papel do IGF-I é hoje considerado relevante a nível dos órgãos esteroidogénicos, dada a sua função mitogénica e sobretudo o seu papel na regulação da esteroidogénese a nível do córtex

supra-renal do homem, boi e rato (Mesiano e col., 1993; Viard e col., 1993, Penhoat e col., 1994; Weber e col., 1997). De facto, são atribuídas ao IGF-I a indução e manutenção de algumas funções diferenciadas a nível das células de Leydig do testículo, granulosas do ovário e adrenocorticais (Bergh e col., 1991; Penhoat e col., 1994), sugerindo um papel local relevante neste tipo celular específico, e ainda corroborado pelo facto de ser sintetizado no tecido adrenocortical e neste ser possível a sua identificação em relativa abundância assim como o seu mRNA (Hansson e col., 1988; Mesiano e col., 1993; Ho e Vinson, 1995). Também de particular interesse, é de salientar que, em situações de proliferação adrenocortical induzida por enucleação ou supra-renalectomia unilateral, este peptídeo é segregado pelo tecido adrenocortical, o mesmo acontecendo após estimulação crónica com ACTH e angiotensina II (Penhoat e col., 1989; Jackson e col., 1991). Tendo em conta a elevada expressão de mRNA para o IGF-I na zona glomerulosa após estimulação com ACTH ou restrição de sódio (Ho e Vinson, 1995), e a presença de receptores de IGF-I nas glândulas supra-renais do homem, boi e rato (Penhoat e col., 1988; Shigematsu e col., 1989; Arafah, 1991; Weber e col., 1995, 1997), a sua acção local nos mecanismos de proliferação compensatória, regeneração e nomeadamente na diferenciação, sai reforçada.

O FGF-2 é um potente factor mitogénico para as células adrenocorticais (Gospodarowicz e col., 1977, 1986; Hornsby e Gill, 1978), e, curiosamente, a sua expressão está aumentada na zona glomerulosa e medula do rato quando submetido a supra-renalectomia unilateral (Basile e Holzwarth, 1993, 1994; Holzwarth, 1995), sugerindo um papel deste peptídeo na regeneração supra-renal compensatória.

A este respeito, também as conexões nervosas parecem estar implicadas no crescimento supra-renal compensatório (Dallman e col., 1976; Gragg e Soliman, 1993). De facto, métodos imunohistoquímicos mostraram a presença de diversos neuropeptídeos nos nervos do córtex supra-renal (Holzwarth, 1984, 1988; Holzwarth e col., 1987; Malendowicz, 1993; Vinson e col., 1994b; Toth e Hinson, 1995), com especial relevo no córtex externo, condicionando suporte para uma evidência de regulação parácrina. Na verdade, quer o neuropeptídeo Y quer o VIP estimulam cronicamente

o crescimento da zona glomerulosa e a secreção de aldosterona (Mazzocchi e col., 1987, 1993; Rebuffat e col., 1988).

No entanto, e apesar dos inúmeros factores com papel importante na morfofisiologia supra-renal, os aspectos referentes aos mecanismos de regeneração e diferenciação adrenocortical com ênfase na sua zonação morfológica e funcional necessitam de um maior esclarecimento, obrigando à criação de modelos experimentais que permitam uma análise dinâmica dos processos biológicos que caracterizam estes mecanismos.

## 3. Objectivos do trabalho e metodologia

O projecto de base da presente dissertação, englobado num grupo de investigação científica básica sobre a morfofisiologia da glândula supra-renal, visa aprofundar os conhecimentos sobre a dinâmica da regeneração e diferenciação glandular utilizando como modelo de proliferação o autotransplante da glândula no rato Wistar. Este animal é de fácil obtenção, havendo grande experiência na sua utilização no Instituto de Histologia e Embriologia da Faculdade de Medicina do Porto. A ideia inicial deste trabalho surgiu em função do conhecimento retrospectivo e confirmação por experimentação pessoal, que o autotransplante desta glândula, no rato, é facilmente exequível e apresenta alta taxa de sucesso, sendo este definido pela sobrevivência do animal sem necessidade de suplementação de corticosteróides, juntamente com a confirmação morfológica e bioquímica da presença de tecido adrenocortical regenerado. Evidentemente, o conhecimento deste facto aliado aos registos da alta taxa de insucesso observada no autotransplante da glândula supra-renal humana, foram o grande motivo que nos levou a desenvolver o presente modelo exposto nesta dissertação. Desta forma, procurou-se esclarecer ou definir uma possível explicação para os correntes achados a nível do autotransplante no homem, com vista à obtenção de dados passíveis de alargar as perspectivas actuais na prática clínica. O estudo em animal experimental mereceu todo o nosso interesse, tanto mais que de acordo com a revisão efectuada, o modelo em causa permite o estudo das modificações observadas no tecido glandular adrenocortical sem intervenção da medula supra-renal, contribuindo para o estudo da regulação local do tecido regenerado, incluindo os mecanismos de zonação e o potencial esteroidogénico.

Um dos aspectos originais presentes no trabalho prende-se com a escolha do local de autotransplante (tecido celular subcutâneo da região dorsal do rato), e cuja vantagem imediata em relação aos locais previamente descritos se baseia simplesmente na mais rápida técnica de execução juntamente com uma maior facilitação do procedimento de exérese cirúrgica do enxerto, sempre que necessário, dada a simplicidade do acesso pela sua colocação superficial. Adicionalmente, e em termos de viabilidade e critérios de regeneração, este modelo permitiu acompanhar paralelamente e sem diferenças significativas, os procedimentos de regeneração observados com outros métodos.

Inicialmente, e dada a utilização de um novo processo de implantação local, efectuámos uma cuidadosa avaliação dos procedimentos de regeneração e diferenciação em termos morfológicos e bioquímicos, tendo sido estes os objectivos do primeiro trabalho. Com a utilização do microscópio de luz e electrónico confirmámos achados prévios no campo da regeneração adrenocortical e avaliámos cuidadosamente, nas fases precoces do desenvolvimento, os processos de proliferação celular utilizando o método da autorradiografia com $^{3}$H-timidina. Esta monitorização morfológica foi acompanhada por doseamentos hormonais plasmáticos aquando da recolha do sangue dos animais durante o sacrifício. Assim, foi doseado o glicocorticóide mais importante do rato – a corticosterona — o que nos permitiu verificar o início da actividade esteroidogénica e proceder à analogia deste facto com o aparecimento das características citoplasmáticas ultrastruturais das células secretoras de esteróides.

Os dois trabalhos seguintes procuram alargar o campo explorado, utilizando o mesmo modelo, mas submetendo o enxerto à acção de substâncias com potencial mecanismo modulador no córtex supra-renal. Baseados na actividade funcional demonstrada por alguns peptídeos de origem vascular, a endotelina-1 foi por nós escolhida dado o seu efeito na estimulação da secreção de aldosterona e pelo seu possível papel na regulação morfofuncional da zona glomerulosa (Hinson e col., 1991). Os efeitos deste peptídeo foram estudados em microscopia de luz e electrónica, e os

doseamentos plasmáticos foram ampliados incluindo o doseamento de aldosterona e a actividade da renina. Perante estes procedimentos tornou-se obviamente necessária uma análise mais específica dos tipos celulares envolvidos no autotransplante, tendo sido utilizados métodos mais refinados de monitorização da zonação adrenocortical. Para tal foi utilizado o anticorpo monoclonal "IZAb" produzido por G. P. Vinson da Universidade de Londres, que se revelou de grande utilidade pela sua capacidade discriminatória. Este anticorpo reage com um antigénio específico da zona interna do córtex supra-renal (zona fasciculada e zona reticular), e está ausente da zona glomerulosa (Laird e col., 1988), permitindo uma identificação inequívoca dos tipos celulares observados no processo de diferenciação e disposição zonal adrenocortical após autotransplante, disposição essa de muito difícil avaliação por outros métodos, nomeadamente por microscopia de luz, e com sérias dificuldades por microscopia electrónica dada a ausência de orientação espacial.

O último trabalho procura descrever as modificações observadas no desenvolvimento dos enxertos adrenocorticais após a administração crónica de factores de crescimento, nomeadamente o factor básico de crescimento fibroblástico (bFGF, FGF-2) e o factor de crescimento de tipo insulínico I (IGF-I), factores com actividade comprovada na promoção da proliferação celular e esteroidogénese a nível das células corticais glandulares no rato (Basile e Holzwarth, 1993; Penhoat e col., 1994). Tal como após a utilização de endotelina-1, o principal objectivo deste trabalho é clarificar as alterações celulares e zonais observadas no contexto da regulação adrenocortical exercida por estes factores na ausência de medula, com particular atenção aos mecanismos de diferenciação celular, utilizando os mesmos métodos dos trabalhos anteriores nomeadamente o estudo imunohistoquímico com "IZAb".

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# PUBLICAÇÕES

# PUBLICAÇÃO I

*Autotransplantation of the adrenal cortex: a morphological and autoradiographic study*

THE ANATOMICAL RECORD 232:262–272 (1992)

# Autotransplantation of the Adrenal Cortex: A Morphological and Autoradiographic Study

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*ABSTRACT* A morphological and autoradiographic study was made of the adrenal gland of adult male rats after autotransplantation. The simple technique involved placement of pieces of the adrenal gland in a dorsal plane between the skin and muscle. Animals for morphological studies were sacrificed at 2, 3, 4, 7, 15, 30, 90, and 180 days after autotransplantation. Those for autoradiographic studies were sacrificed at 2, 3, 7, and 15 days after autotransplantation, with $^3$H-thymidine being administered intraperitoneally 2 h before sacrifice. Sham-operated animals were used as controls. The majority of glandular adrenal cells suffered necrosis in the first days (2 and 3) after autotransplantation. Up until 15 days and after revascularization, morphological features of the cells were compatible with protein synthesis exhibiting a developed RER, scarce SER, and mitochondria with tubular and lamellar cristae. These data may correspond to a proliferative phase of glandular cells. At day 15, cells showed morphological signs of steroidogenic activity (mitochondria with vesicular cristae, increase of SER), and at day 30, an increased number of microvilli were seen. Between 30 and 90 days zonation of the adrenal was evident with glomerulosa, fasciculata, and reticular zones readily apparent. The quantitative analysis showed a significant increase of the volumetric density of mitochondria and microvilli between the days 7 and 30.

Autoradiographic studies showed an intense labelling of fibroblast-like cells at days 2 and 3 and of glandular cells at days 7 and 15, which was confirmed by the quantitative studies.

Corticosterone in autotransplanted animals decreased during the first 15 days, but after 30 days the values were similar to controls. The model reported here seems to be good for study of the regeneration of the adrenal gland and can be a simple, useful, and reproducible method for adrenal transplantation.

Daily steroid replacement is required after total adrenalectomy for diseases such as Cushing's syndrome or pheochromocytoma when both adrenal glands are affected (Saxe and Connors, 1985). This treatment is uncomfortable, and it can lead to undesirable side effects. Preservation of endogenous glucocorticoid and mineralocorticoid secretion by the autotransplantation of the adrenal gland would be the ideal treatment (Saxe and Connors, 1985).

Adrenal autotransplantation has already been done in laboratory animals. In 1898 (Poll, noted in Wyman and Suden, 1932), the first adrenal viable grafts were accomplished. Following that, adrenals from rats, mice, guinea pigs, frogs, rabbits, and cats have been autotransplanted and the grafts placed in different organs such as the brain, ear, eye, thyroid, testis, portal tract (Penney et al., 1963), spleen (Belloni et al., 1982), and musculus gracilis (Belloni et al., 1990). The success of the autotransplant was morphologically observed and the development of adrenal regeneration has been biochemically evaluated either through the assessment of corticosterone, cholesterol, and ascorbic acid in the adrenals and plasma (Skelton, 1959) or by studying corticosterone rhythm in basal and stress conditions (Wilkinson et al., 1981; Murakami and Takahashi, 1982; Engeland, 1986).

In spite of these studies, human adrenal autotransplantation has not yet been possible since it is extremely resistant to transplantation. We have, therefore, decided to re-examine this problem developing a simple, easily reproducible, autotransplant model on rats, with the intention of studying adrenal regeneration over a long period and the origin of the regenerated cells in order to apply the model to human beings. Also, the model may be useful in further studies on paracrine adrenal regulation.

Thus we studied: (1) the ultrastructure of adrenal grafts in autotransplanted rats between 2 and 180 days, (2) the type of proliferative cells in early stages of graft regeneration, between 2 and 15 days, after $^3$H-thymidine administration, and (3) corticosterone levels during autotransplant regeneration.

## MATERIALS AND METHODS

Twenty-six male Sprague-Dawley rats, weighing approximately 200 g, were derived from the colony of the

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Gulbenkian Institute of Science, Oeiras, Portugal, and divided into two experimental groups.

For morphologic studies by light and electron microscopy, 22 animals (first group) were autotransplanted and sacrificed at 2, 3, 4, 7, 15, 30, 90, and 180 days. For autoradiographic studies, four rats (second group) were autotransplanted, injected intraperitoneally with $^3$H-thymidine (1 μCi/gr body weight) 2 h before sacrifice, which took place at 2, 3, 7, and 15 days.

After anesthesia with Nembutal (0.1 ml/100 g body weight), the animals of both groups underwent total adrenalectomy. The adrenals were placed in a 0.9% sterile saline solution and cut in small pieces measuring 1–2 mm each. Between 10 and 20 pieces were autotransplanted immediately under the skin of the dorsal region using the incision previously made. The incision was sutured with "cat gut" and silk. Some animals were "sham operated" without removal of adrenal glands and served as controls.

All animals were fed a commercial diet and provided 0.9% saline solution during the first 90 days and subsequently with water until sacrifice, which was performed by decapitation after anesthesia with nembutal. Necropsy was carried out on all animals in order to search for accessory adrenals.

Adrenal grafts and adrenal glands from control animals were fixed in Bouin's liquid for 24 h, formol for 48 h, or Zenker's for 48 h, and paraffin embedded. Pieces of adrenal grafts were also fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, at 4°C for 2 h, postfixed in 1% osmium tetroxide in veronal-acetate buffer, pH 7.3, at 4°C for 2 h, and Epon embedded. Sections of 1 μm in thickness were stained with methylene Blue-azur II for light microscopy (Richardson et al., 1960). Ultrathin sections were stained with alcoholic uranyl acetate and lead citrate and examined in a Jeol 100 B electron microscope.

For autoradiographic studies by light and electron microscopy, we used the techniques previously described (Magalhães et al., 1986). In short, sections of 1 μm thickness were placed on glass slides, stained with periodic acid-Schiff plus Harris hematoxylin, and dipped into Ilford $K_5$ nuclear emulsion. After an exposure period of 12, 26, or 33 days at 4°C, radioautographs were developed in Kodak D-170 for 6 min and fixed in 24% sodium hyposulfite for 2 min at 18°C.

For ultrastructural autoradiographic studies, ultrathin sections were placed onto nitrocellulose-coated glass slides and dipped in Ilford $L_4$ nuclear emulsion. After an exposure period of 39, 63, or 90 days at 4°C, radioautographs were developed in D-19b for 3 min at 20°C and fixed in 24% sodium hyposulfite for 2 min at 18°C, after which sections were stained with uranyl acetate (15 min) plus lead citrate (10 min) (Reynolds, 1963).

Morphometric studies were performed using adrenal grafts with 7, 30, and 90 days, from three rats for each time period. Gold ultrathin sections from three Epon blocks per animal were cut and 10 micrographs per rat were taken at random at 6,000x, so that 30 micrographs were studied for each animal group. A square test—the sides measured 14 cm containing 112 lines of constant length (1 cm), arranged in 16 equidistant and parallel rows, with the distance between the end points of the lines in every direction also 1 cm—was placed on photographic prints that had been enlarged to 18,000x over zones with visible extracellular space. The number of points that fell on the mitochondria and on the interdigitating cell extensions—microvilli—was used for point-counting volumetry. The number of points ($P_i$) for these structures was transformed into volumetric density in agreement with the formula $V_{vi} = P_i/P_T$ (Weibel, 1973) expressed as a percentage ($P_T$ is the total number of test points). The average values were compared by the Student t-test.

The number of labelled cells was counted at 700x under oil immersion in 10 fields per gland. Only nuclei with five or more grains were counted as labelled nuclei. In each field, the total number of glandular and fibroblast-like cells was counted in order to calculate the percentage of labelled cells.

The number of silver grains in cell nuclei was counted over a field outlined by an ocular grid by using the immersion oil objective lens. The grid consisted of a square measuring 225 $\mu^2$, which was placed over the nucleus of glandular or fibroblast-like cells. At the three different times, silver grains were counted on 50 nuclei of each cellular type and the results were expressed as number of silver grains per 1,000 $\mu^2$ of tissue. The mean background count measured outside the sections was deducted from the mean of the nuclear measurements. The average values were compared by the Student t-test.

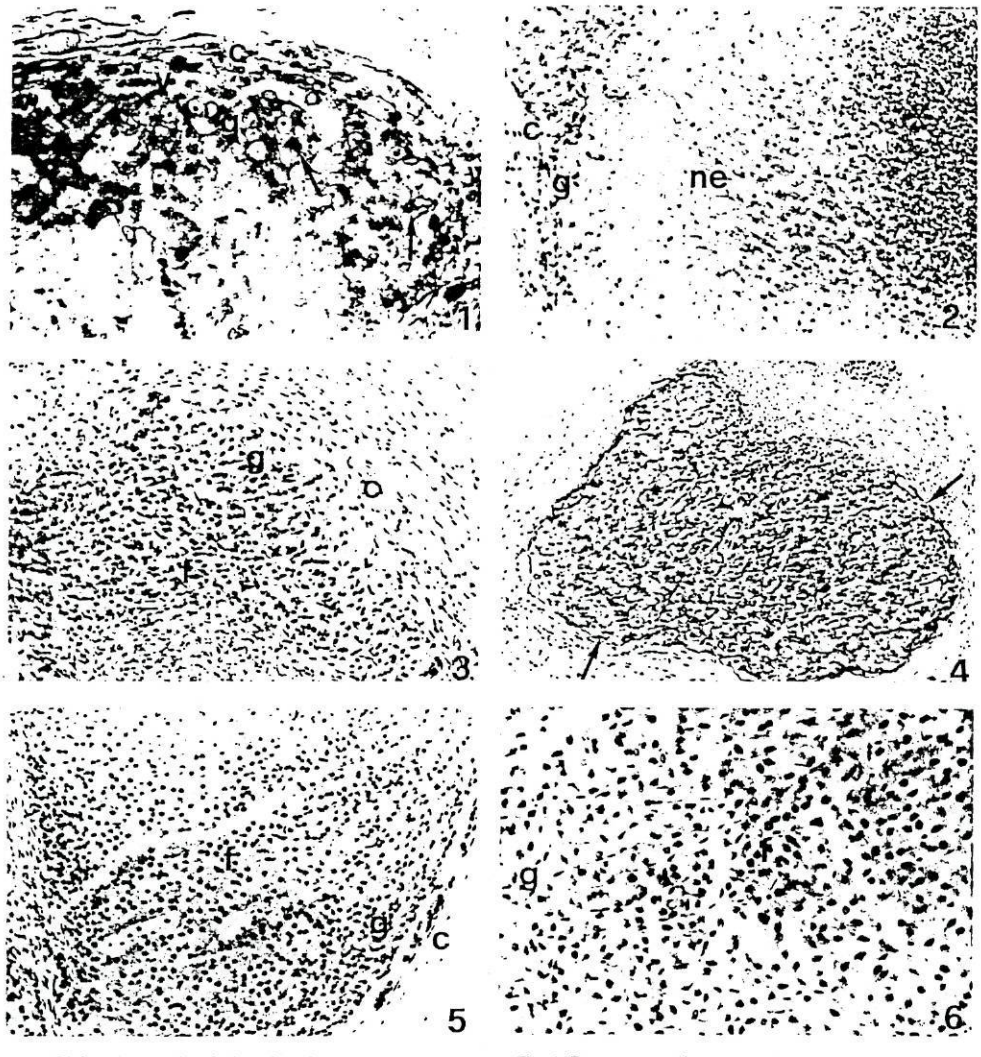
For the assessment of corticosterone in the plasma of autotransplanted and sham-operated animals, the fluorometric method of Mejer and Blanchard (1973) was used. The fluorescence was read on a spectrofluorometer at an excitation of 470 nm and an emission of 520 nm. These determinations were done in duplicate. The average values were compared by the Student t-test.

## RESULTS

### *Light Microscopy*

Adrenal gland structure was identical in both normal and sham-operated animals.

Forty-eight hours after autotransplantation, the adrenal fragments (capsule included) showed some architectural changes (Fig. 1). The capsule became oedematous and the cells underneath it (subcapsular cells) were glomerulosa-like, with large and oval nuclei. The cytoplasm contained a large number of lipid droplets, but mitoses were not found. The remaining parenchyma exhibited necrosis (Fig. 1). The blood supply was seen penetrating throughout the capsule, directing to the graft center. Fibroblast-like cells, polymorphonuclear leucocytes, lymphocytes, and macrophages were present in the capsule and subcapsular zone. At 72 hours, the periphery and center of the graft became well defined (Fig. 2). The periphery of the graft exhibited the capsule and zona glomerulosa with a large number of capillary vessels, whereas the center exhibited necrotic tissue, filled with a large number of polymorphonuclear leucocytes, lymphocytes, and macrophages (Fig. 2). The quantity of lipid droplets in the glomerulosa cells seemed to be smaller than at 48 hours. Between 4 and 7 days the necrotic center disappeared and became occupied by more or less individualized cell cords separated by fibroblast-like cells. These cell cords seemed to grow centripetally. Zonation and an adrenal medulla were not seen. At 15 days,




Figs. 1–6. Light micrographs of adrenal grafts.

Fig. 1. Postautotransplantation—2 days. Note the blood vessels (v) and the large number of lipid droplets (arrows). c—capsule; g—subcapsular or glomerulosa cells. × 225.

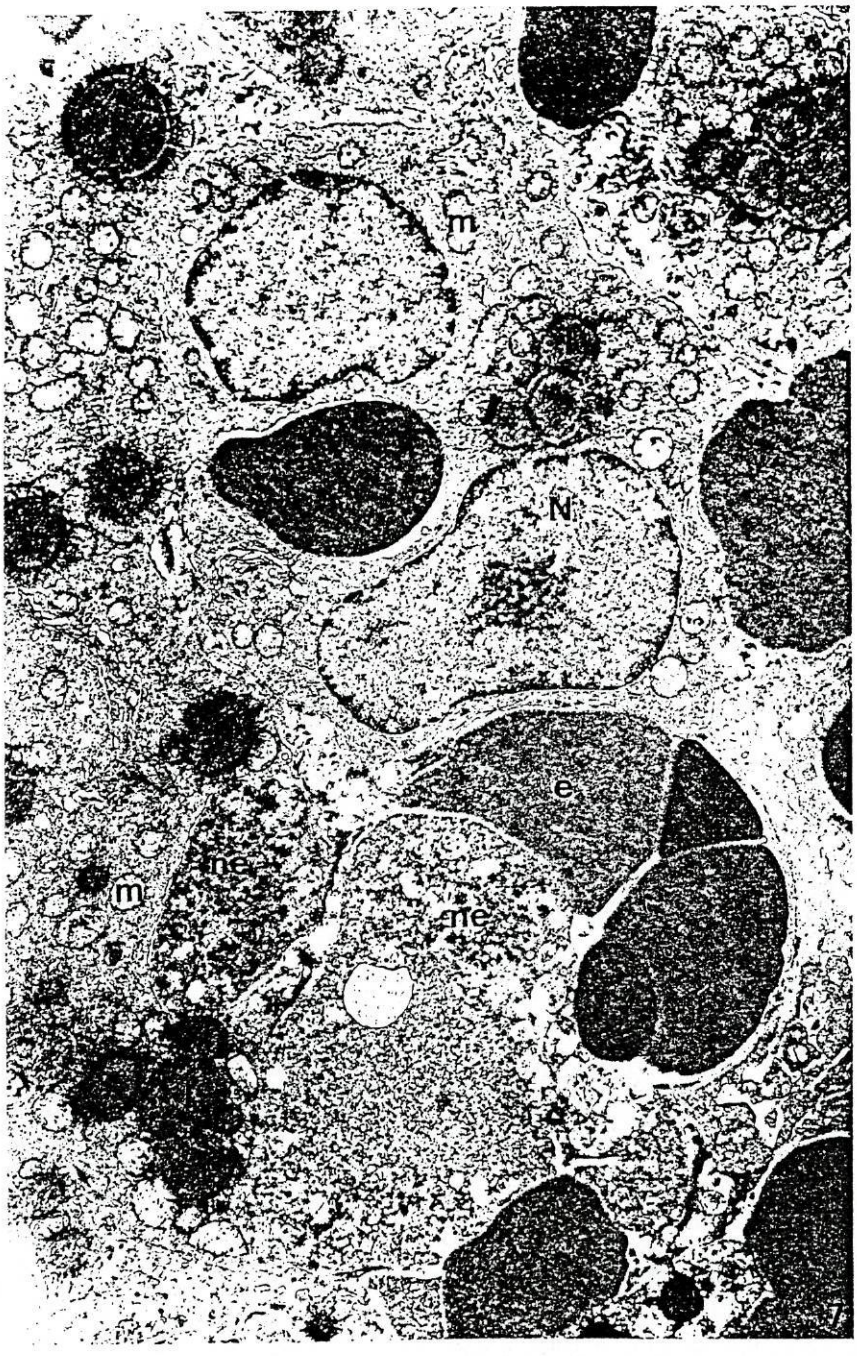
Fig. 2. Postautotransplantation—3 days. Capsule (c) and subcapsular or glomerulosa cells (g) are visible on the left; the center exhibits necrosed tissue (ne); a dense number of polymorphonuclear leucocytes are seen on the right (*). × 150.

Fig. 3. Postautotransplantation—15 days. Zonation begins to appear. Note the capsule (c), zona glomerulosa (g) and zona fasciculata (f). × 150.

Fig. 4. Postautotransplantation—30 days. "Nests" of adrenal cells are surrounded by the capsule (arrows). × 70.

Fig. 5. Postautotransplantation—90 days. Adrenal cortical architecture appears normal with glomerulosa (g), fasciculata (f) and reticularis (r) zones. × 225.

Fig. 6. Postautotransplantation—180 days. Glandular architecture is identical to adrenals of normal animals. f—fasciculata; g—glomerulosa. × 150.




Figs. 7–11. Electron micrographs of adrenal grafts.

Fig. 7. Subcapsular glomerulosa cells, 2 days postautotransplantation. Note the large number of lipid droplets (l) and mitochondria (m) with tubular and lamellar cristae. Blood vessels with numerous erythrocytes inside (e) separate normal cells from necrosed tissue (ne). × 9,000.

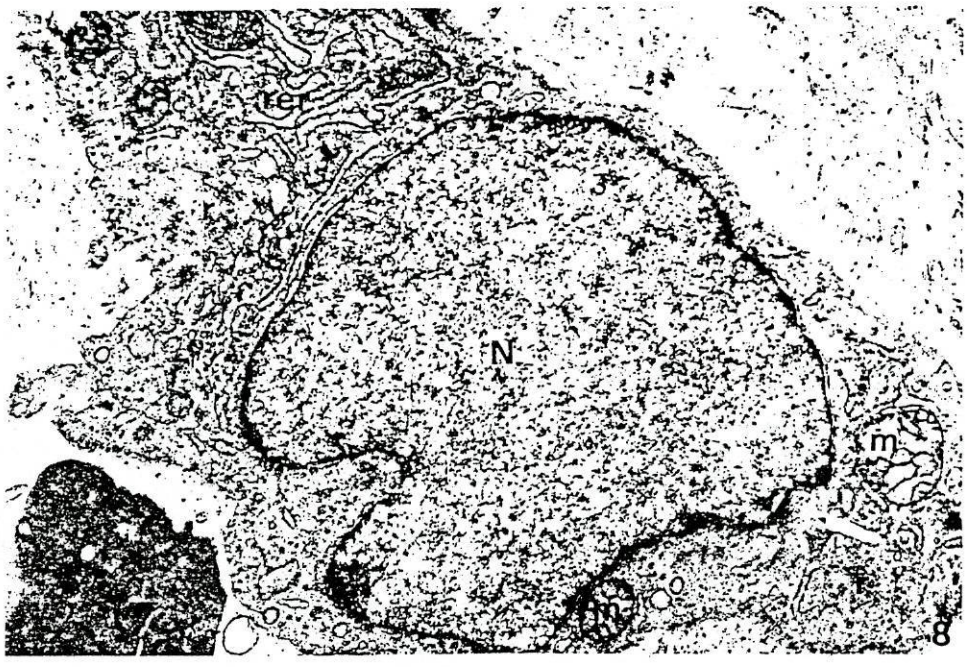



Fig. 8. Postautotransplantation—7 days. Note mitochondria (m) with atypical tubulo-vesicular cristae. Numerous profiles of rough endoplasmic reticulum (rer) and large number of free ribosomes (r) are seen. N—nucleus. × 16,800.

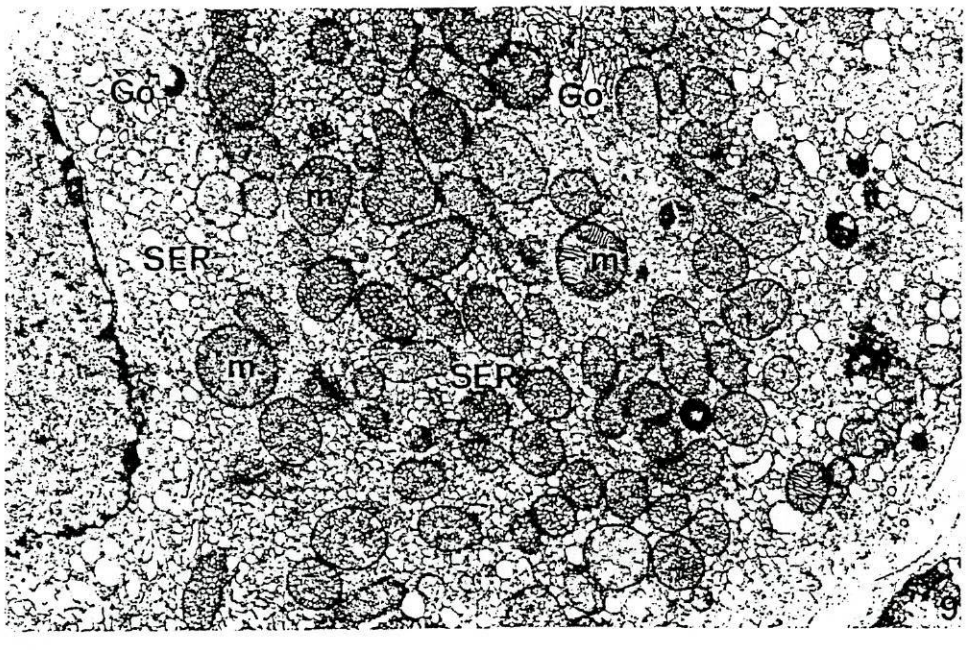
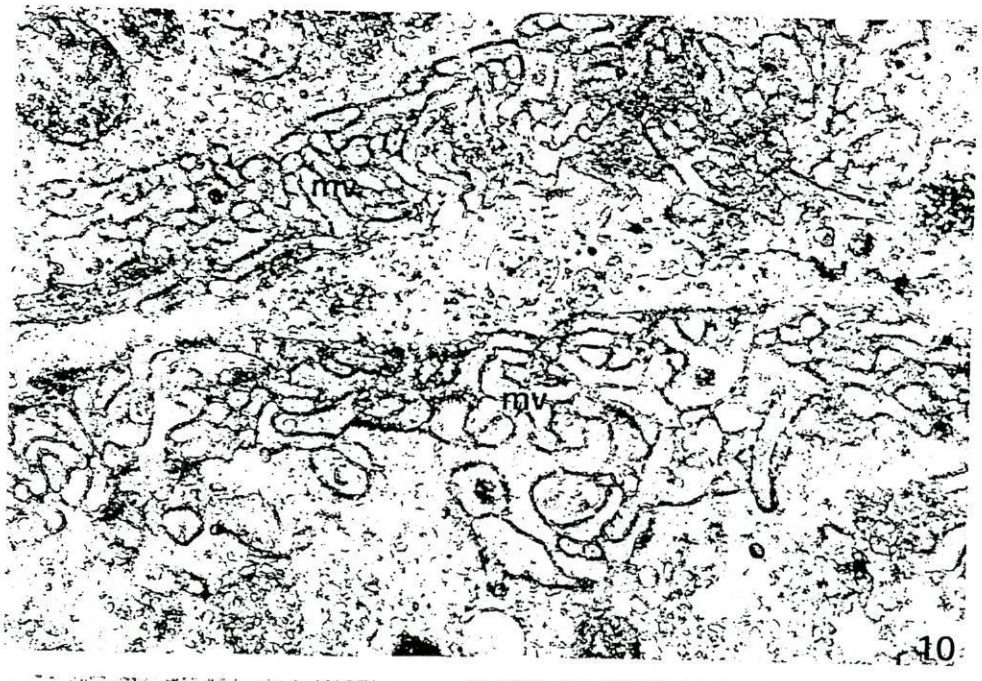



Fig. 9. Postautotransplantation—15 days. Adrenal cells showed mitochondria (m) with vesicular and tubulo vesicular cristae. Note the smooth endoplasmic reticulum (SER). Go—Golgi complex. 12,000.



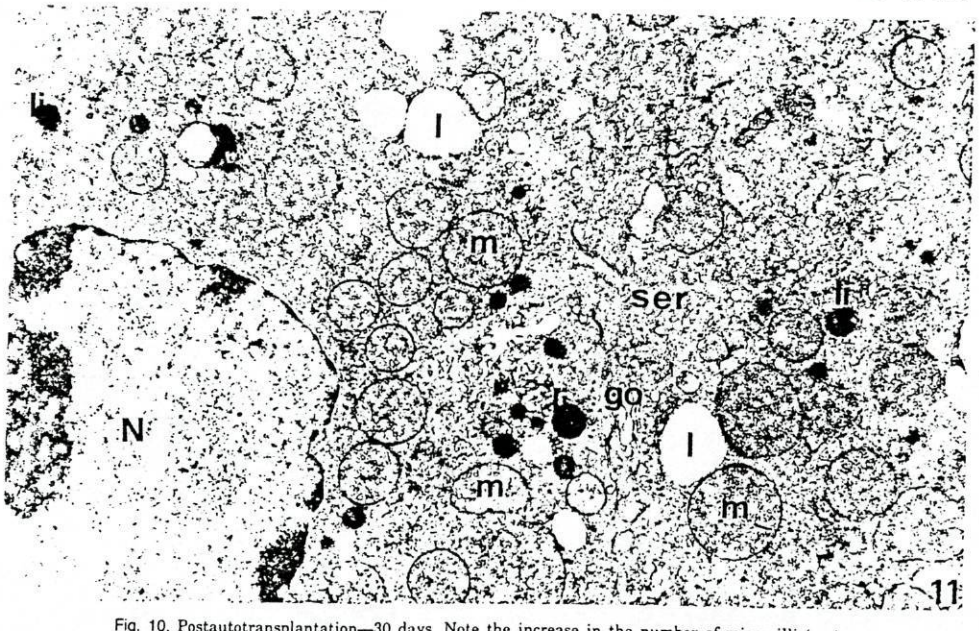




Fig. 10. Postautotransplantation—30 days. Note the increase in the number of microvilli (mv). × 25,200.

Fig. 11. Postautotransplantation—180 days. The ultrastructure is indistinguishable from normal adrenal cells. go—Golgi complex; l—lipid droplets; li—lysosomes; m—mitochondria; N—nucleus; ser—smooth endoplasmic reticulum. × 13,440.

parenchymal architecture changed and the beginning of zonation could be observed, with the appearance of a zona glomerulosa and zona fasciculata (Fig. 3). A zona reticularis and adrenal medulla were not found. There was obvious cell growth, proliferation, and connective tissue occupying the graft center (Fig. 3). Thirty days after the autotransplantation, "nests" of glandular cells were observed surrounded by the capsule (Fig. 4). These "nests" were arranged in the glomerulosa and fasciculata zones. Sometimes fibrous septa arising from the connective tissue of the capsule could be seen, dividing the glandular parenchyma. At 90 days, the capsule was well defined and the three zones of the adrenal cortex were visible (Fig. 5). Fibrous tissue was present occupying the graft center. At 180 days after the autotransplant, cortical architecture was identical in all aspects to adrenal glands of normal animals (Fig. 6).

### *Electron Microscopy*

Adrenal gland ultrastructure was identical in both normal and sham-operated animals. At 48 hours following transplantation, the subcapsular glomerulosa cells exhibited large, oval nuclei with patches of condensed chromatin placed close to the inner leaflet of the nuclear envelope. The cytoplasm contained a large number of lipid droplets close to each other (Fig. 7). Free ribosomes were also numerous. The Golgi complex exhibited stacks of 3–4 cisternae with smooth and coated vesicles. Mitochondria appeared with tubular cristae; however, they were found to periodically have lamellar cristae (Fig. 7). Contiguity of lipid droplets and mitochondria was frequently found. Smooth and rough endoplasmic reticulum profiles were scarce. Blood vessels were numerous with a large number of erythrocytes in their lumina (Fig. 7). These vessels often separated normal cells from necrosed tissue (Fig. 7). At 3 and 4 days, the cell ultrastructure was similar to that at 2 days. Apparently, the lipid droplets were smaller, and there was an increase in the rough endoplasmic reticulum profiles. Mitosis were seen in small number. Seven days after autotransplantation, cells presented numerous profiles of rough endoplasmic reticulum as well as a large number of free ribosomes. Mitochondria presented "atypical" tubulovesicular cristae (Fig. 8). The number of lipid droplets was smaller than previously observed. At 15 days, the cell ultrastructure was similar to that of the normal animals, with numerous profiles of smooth endoplasmic reticulum and a few of rough endoplasmic reticulum. Mitochondria exhibited vesicular and tubulovesicular cristae. Features of cells secreting steroids were present (Fig. 9). At 30 days the ultrastructure of the glandular cells was similar to that in sham-operated animals. They exhibited mitochondria with vesicular cristae, smooth endoplasmic reticulum, and lipid droplets. A remarkable increase in the number of microvilli of the glandular cells occurred in this period (Fig. 10). At 90 and 180 days (Fig. 11) after autotransplantation, the fine structure of the glandular cells was indistinguishable from normal adrenal glands; however, the number of microvilli seemed smaller than that observed at 30 days.

Quantification of the relative volume of mitochondria and microvilli showed a significant increase of the volumetric density of the mitochondria between the 7th and 30th day (Table 1); concerning the microvilli, there was an highly significant increase of the same parameter until the 30th day, which was then followed by a remarkable decrease (Table 1).

**TABLE 1. Variations of volumetric density ($\bar{x} \pm SE$) of mitochondria and microvilli with the age of autotransplantation**

| Day | Mitochondria | Microvilli |
|---|---|---|
| 7 | 20.66 ± 1.46 | 5.24 ± 0.46 |
| p 7 days vs 30 days | < 0.01 | < 0.001 |
| 30 | 28.09 ± 1.35 | 18.49 ± 1.58 |
| p 30 days vs 90 days | n.s. | < 0.001 |
| 90 | 28.23 ± 2.07 | 9.14 ± 1.20 |
| p 7 days vs 90 day | < 0.05 | < 0.05 |

### *Autoradiographic Studies in LM and EM*

Autoradiographic studies were performed in autotransplanted animals sacrificed at 2, 3, 7, and 15 days. Morphological studies in light and electron microscopy revealed identical aspects to the previously described observations in the corresponding periods. Autoradiograms by light microscopy showed silver grains on the fibroblast-like cells at 2 and 3 days (Figs. 12, 13) and an intense labelling of the glandular cells located under the fibrous capsule cells at 7 and 15 days (Figs. 14, 15). Ultrastructural autoradiograms showed silver grains over the nuclei of fibroblast-like cells at 2 and 3 days of autotransplantation (Fig. 16) and confirmed the light microscopy observations with the silver grains located most over the glandular cells nuclei after 7 and 15 days of autotransplant (Fig. 17). Cells with ultrastructural features of adrenal medullary cells were observed 7 days after the autotransplant (Fig. 18).

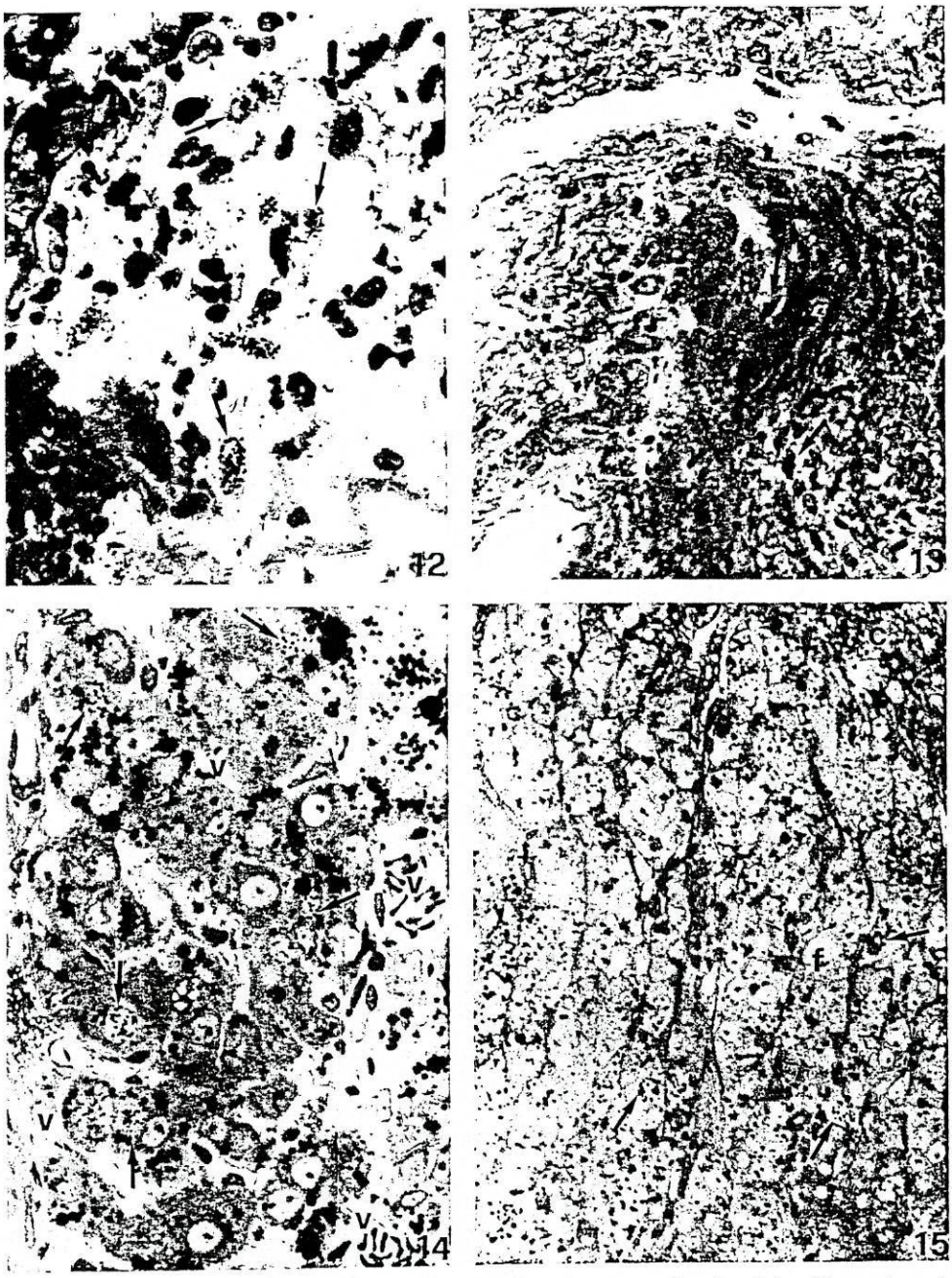
The study of labelled cells showed a great percentage of labelled fibroblast-like cells at day 2 followed by a significant decrease, which was opposite to that seen in the adrenal glandular cells (Table 2). Also the number of silver grains over the cell nuclei decrease in fibroblast-like cells and increased in the adrenal glandular cells (Table 2).

### *Corticosterone Assessment*

Until the period of 15 days after autotransplantation, values for plasma corticosterone were less than 0.05 μg/ml. After this period, we found the following average values: 0.19 μg/ml at 30 days; 0.15 μg/ml at 90 days, and 0.19 μg/ml at 180 days. The average value in the sham-operated animals was 0.20 μg/ml (Table 3).

## DISCUSSION

When adrenal gland autotransplantations were first performed some years ago, the basic purpose was to define the gland regeneration process (Ingle and Higgins, 1938; Greep and Deane, 1949; Brenner et al., 1953; Penney et al., 1963; Seki et al., 1969; Taki and Nickerson, 1985). Autotransplantation success was achieved above all in rodents, which is why rats and mice are the animals of choice in most experiments (Wyman and Suden, 1932; Baxter, 1946; Greep and Deane, 1949; Brenner et al., 1953; Penney et al., 1963; Murakami and Takahashi, 1982; Saxe and Connors,



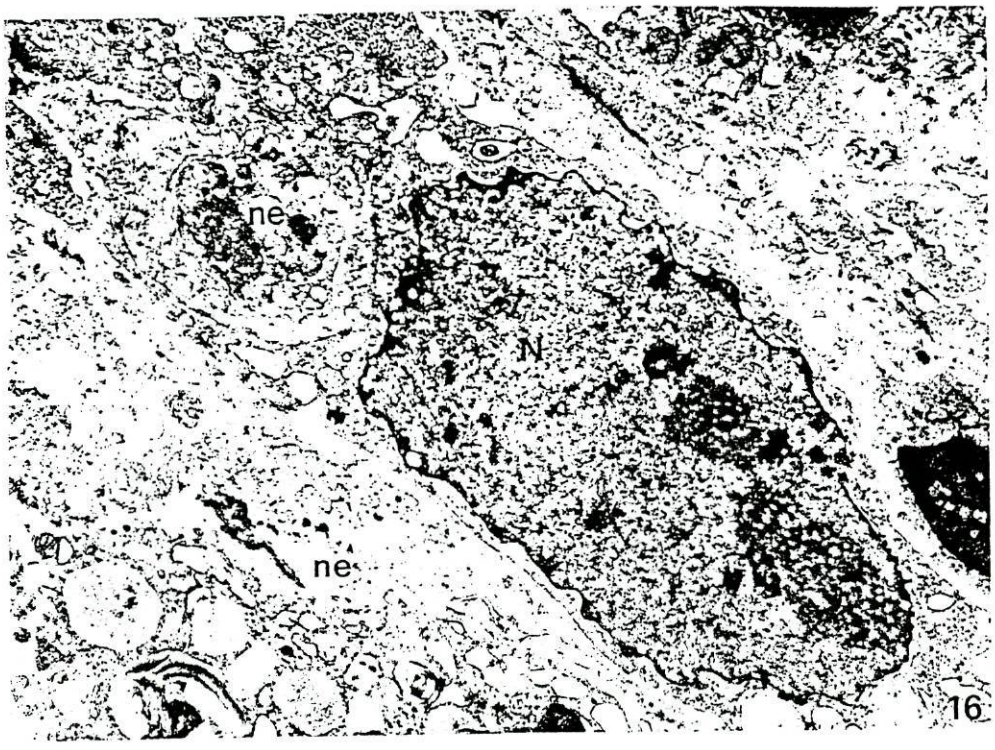

Figs. 12–15. LM autoradiographs of adrenal grafts after [$^3$H] thymidine administration. Development in Kodak D-170.

Fig. 12. Postautotransplantation—2 days. Silver grains overlay nuclei of the fibroblast-like cells (arrows). Exposure 26 days. × 640.

Fig. 13. Postautotransplantation—3 days. Nuclei of fibroblast-like cells are strongly labelled (arrows). Exposure 26 days. × 405.

Fig. 14. Postautotransplantation—7 days. Silver grains are located over glandular cell nucleus (arrows). v—blood vessels. Exposure 33 days. × 630.

Fig. 15. Postautotransplantation—15 days. Note the labeling of glandular cells (arrows), mainly in zona fasciculata (f). Exposure 26 days. × 405.




Figs. 16–17. EM autoradiographs of adrenal grafts after [$^3$H]-thymidine administration. Development in Kodak D19b.

Fig. 16. Necrosis zone (ne), 2 days postautotransplantation. Note the silver grains over the nucleus (N) of fibroblast-like cells. Exposure 63 days. × 10,800.

1985; Engeland, 1986). Nevertheless, the usual implantation sites (ovary, muscle, etc.) (Ingle and Higgins, 1938; Brenner et al., 1953) do not seem to be very practical or easily performed when one wishes to transfer these studies to humans. That is why we made use of a simple technique by placing the adrenal fragments in a superficial, easily approachable site (which is under the skin of the dorsal region) using the same incision previously made to perform the bilateral adrenalectomy.

Morphological studies allowed us to establish a series of changes that can be schematized in this way: tissue necrosis, particularly of the inner layers, 2–3 days after the autotransplant, appearance of large subcapsular glomerulosa cells filled with lipid droplets, scarce smooth endoplasmic reticulum profiles, numerous free ribosomes and rough endoplasmic reticulum, and mitochondria with lamellar and tubular cristae. After the 7th day, cells acquired some features of steroid-producing cells with the appearance of a large number of mitochondria with tubular cristae and with a decrease in the number of lipid droplets; at 30 days, the graft cells seemed normal adrenal cells. Simultaneously, biochemical studies showed the acquisition of functional capacity by autotransplanted cells since plasma corticosterone measurements in the animals, after tissue regeneration, were similar to those found in sham-operated animals. Recent studies using HPLC data (Belloni et al., 1990) also showed that steroidogenic capacity of autotransplants seems to be more elevated than that of sham-operated animals.

Necropsy, which was carried out in all animals, allowed us to confirm that every time accessory adrenals were present, there was a decrease in the number of viable autotransplanted fragments. This had already been verified by Wyman and Suden (1932). These authors attributed the presence of atrophic fragments to the existence of accessory adrenals or to the presence of glandular fragments, which had not been removed when bilateral adrenalectomy was performed.

Autotransplant regeneration is related to endogenous ACTH stimulation since plasma ACTH concentrations are increased after bilateral adrenalectomy (Wilkinson et al., 1981). The questions are how long does it take for full regeneration to occur and how does regeneration take place? In our experimental model, full regeneration took place between 30 and 90 days and zonation became well defined. In the Ingle and

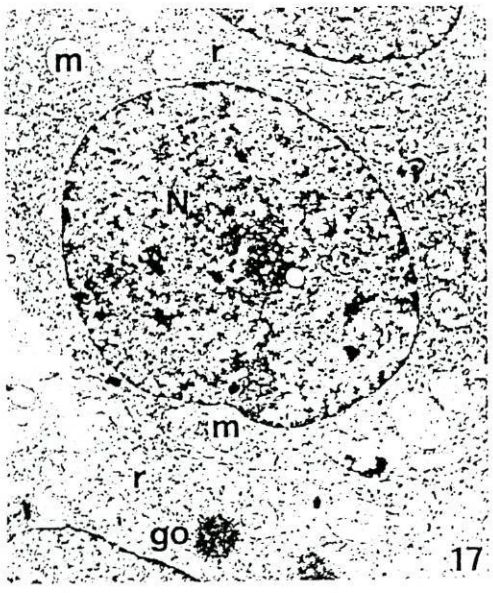



Fig. 17. Adrenal cells, 15 days postautotransplantation. Note the silver grains over the nucleus (N) of glandular cells; mitochondria (m) with typical vesicular cristae. r—free ribosomes. go—Golgi complex. Exposure 39 days. × 9,520.

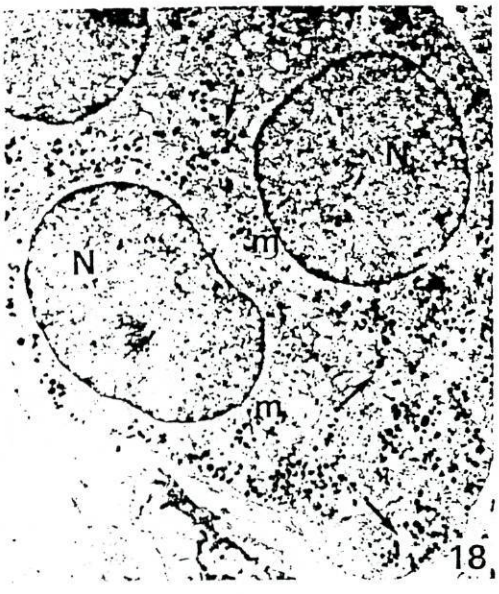



Fig. 18. Electron micrograph of cells with ultrastructural features of adrenal medullar cells, 7 days postautotransplantation; granules (arrows) are dispersed throughout the cytoplasm. N—nucleus. × 7,560.

**TABLE 2. Percentage of labelled cells and number of silver grains in fibroblast-like and glandular cells[1]**

| Day | Labelled cells (% ± SE) | | No grains/1,000 $\mu^2$ ± SE | |
|---|---|---|---|---|
| | Fibroblast-like cells | Glandular cells | Fibroblast-like cells | Glandular cells |
| 2 | 35 ± 1.57(a)[1] | 4 ± 1.86(d) | 156.53 ± 8.80(g) | 125.24 ± 7.60(j) |
| 7 | 8 ± 1.22(b) | 27 ± 2.54(e) | 52.93 ± 3.60(h) | 133.15 ± 6.08(k) |
| 15 | 7 ± 1.22(c) | 34 ± 1.86(f) | 84.22 ± 5.37(i) | 154.75 ± 6.75(l) |

[1]Statistical comparison of the data ($p <$): a vs d—0.001; b vs e—0.001; c vs f—0.001; a vs b—0.001; b vs c—n.s.; a vs c—0.001; d vs e—0.001; e vs f—n.s.; d vs f—0.001; j vs k—n.s.; j vs l—0.001; k vs l—0.01; g vs h—0.001; g vs i— 0.001; h vs i—0.001; g vs j—0.01; h vs k—0.001; i vs l— 0.001.

**TABLE 3. Corticosterone concentrations (μg/ml) in the plasma after autotransplantation**

| Day | Sham operated | p values | Autotransplant |
|---|---|---|---|
| 2/15 | 0.20 ± 0.012 | | * |
| 30 | 0.18 ± 0.012 | n.s. | 0.19 ± 0.029 |
| 90 | 0.21 ± 0.025 | n.s. | 0.15 ± 0.01 |
| 180 | 0.21 ± 0.012 | n.s. | 0.19 ± 0.03 |

* = values lower than 0.05 μg/ml.

Higgins (1938) experimental model and in the model of Brenner et al. (1953), regeneration was completed, respectively, at 30 and 21 days. Revascularization could explain the precocity of the regeneration in Brenner's model since autotransplantation was performed in muscle that is a well-vascularized tissue. Supporting this suggestion we must say that Greep and Deane (1949) studied autotransplantation regeneration using a type of model that preserved the glandular blood circulation (gland enucleation). These authors managed to obtain full regeneration with this model 1 month after the procedure. We achieved full regeneration of our model later than these authors, we believe because the chosen site will require a longer time to induce revascularization of the graft. However, our regeneration time seems precocious enough especially if we think of the advantages of an easy approach site in a clinical management, not in an experimental one.

Concerning the question of how regeneration takes place, autoradiographic studies permitted us to assume that the regeneration process proceeds from the graft periphery. Skelton (1959) reported this theory with different experimental models concerning the actual site from which the new cells were derived. Autoradiographic studies from Seki et al. (1969) with the $^3$H-

thymidine and using the gland enucleation model showed a rapid and complete proliferation before the end of the first month. According to our autoradiographic studies, we admit that regenerated cells arise from supcapsular glomerulosa cells. In fact, we do not admit a capsular source of regenerated cells as is mentioned by other authors (Zwemer et al., 1938; Baxter, 1946) since we did not observe any changes in capsular fibroblasts in relation to their number and ultrastructure. In fact, Greep and Deane (1949) did not obtain in their enucleation studies suggestible data that could permit to affirm the capsule role in adrenal regeneration. The autoradiographic labelling of fibroblast-like cells at 2 and 3 days (Table 2) surrounding the autotransplant seems to represent an attempt to form connective tissue septa dividing viable cells from necrosed tissue. One month after autotransplantation, these septa are present dividing "nests" of glandular cells. Our data suggest, then, that subcapsular glomerulosa cells, which become intensely labelled by $^{3}$H-thymidine at 7 and 15 days (Table 2), are responsible for glandular cell regeneration. These data support the cell migration theory suggested in 1951 by Celestino da Costa. In fact, the zonation theory does not seem to be adjusted to the biologic process of the autotransplant because if initially there was extensive necrosis of glandular cells from the zonulae fasciculata, reticularis and part of the glomerulosa, these zones could certainly not develop a regeneration process. We think that the subcapsular glomerulosa cells will proliferate and undergo a zonal differentiation probably facilitated by the reconstruction of glandular vascularization and its secretory peptides, which might have a role in the control of zona glomerulosa function (Hinson et al., 1990).

Occasionally, the presence of cells with ultrastructural features of the adrenal medullary cells were found at day 7 after autotransplantation. The absence of an adrenal medulla is a current fact described by the majority of authors working in adrenal autotransplantation (Ingle and Higgins, 1938; Skelton, 1959). Nevertheless, Wyman (1928), Turner (1939), and Coupland (1957, 1958) had already referred to its presence. However, the meaning of this fact remains unknown. Regulatory mechanisms common to both the cortex and medulla are being investigated. In fact, recent data brings new findings in this subject, and it is now evident that the products of each of these tissues influence the function of the other (Carballeira and Fishman, 1980; Hinson, 1990).

## ACKNOWLEDGMENTS

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# PUBLICAÇÃO II

*Modulation of autotransplanted adrenal gland by endothelin-1: a morphological and biochemical study*

THE ANATOMICAL RECORD 246:98–106 (1996)

# Modulation of Autotransplanted Adrenal Gland by Endothelin-1: A Morphological and Biochemical Study

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*ABSTRACT* ***Background:*** **Adrenal gland autotransplantation, a model of cortical tissue regeneration, provides the reconstruction of distinct functional and morphological zonae. A morphological and biochemical study of the adrenal gland of adult male rats after autotransplantation and endothelin-1 (ET-1) administration was made.**

***Methods:*** **The technique involved bilateral adrenalectomy and placement of pieces of the adrenal gland in a dorsal plane between the skin and muscle. The animals were killed 90 days after the autotransplantation and 1 hr after intravenous ET-1 administration (0.5 μg/kg body weight). The autotransplanted pieces were removed, fixed, and processed for light and electron microscopic morphologic studies. Trunk blood was collected for steroid assay.**

***Results:*** **Saline-treated control autotransplanted animals showed no remarkable differences in adrenal organization; grafts exhibiting a mass of regenerated cortical tissue were arranged in nests of glandular cells surrounded by a fibrous capsule and intersected by layers of connective tissue. The adrenal medulla was systematically absent. Ultrastructure of ET-1-treated animals revealed an inner area in the graft, consisting mainly of fasciculatalike cells. Cytoplasmic changes were evident, with high variations in mitochondrial size and arrangement. Profiles of smooth endoplasmic reticulum sometimes exhibited evidence of hypertrophy. Glandular cells in the graft outer area (subcapsular) were almost invariably like glomerulosa; however, some of them showed mitochondria with a peculiar arrangement of the cristae. "Hybrid" cells with mitochondria resembling those of the zona reticularis were also observed in the subcapsular environment. ET-1-stimulated animals showed significant increases in plasma corticosterone and aldosterone concentrations.**

***Conclusions:*** **Endothelin-1, previously reported to stimulate acutely the aldosterone secretion by the adrenal zona glomerulosa in the rat, seems to exert a modulator role on the physiology of adrenal autotransplants, their regeneration and secretion.** © 1996 Wiley-Liss, Inc.

**Key words: Autotransplantation, Adrenal cortex, Regeneration, Endothelin-1, Aldosterone**

Regeneration of the rat adrenal can be obtained after autotransplantation of adrenal tissue (Ingle and Higgins, 1938; Skelton, 1959; Saxe and Connors, 1985; Belloni et al., 1990). In a recent paper (Vendeira et al., 1992), we confirmed such adrenal regeneration following autotransplantation from 2 until 180 days. In addition, we observed that after day 30 regenerated cells were surrounded by a capsule from which a few septa detached and penetrated through the grafts. We assumed that regeneration processes took place at the graft periphery, proceeding from subcapsular glomerulosa cells. Furthermore, glomeruldalike cells were reduced to small clusters beneath the capsule or septa, with the greatest cell population of the autograft being faciculatalike cells. In addition, cell cords of irregular shape surrounded by dilated blood vessels (neovascularization) were observed beneath the capsule or septa.

This type of regeneration, particularly the neovascularization at the graft periphery, led us to discuss the role of vascular endothelium in adrenal regeneration. In fact, adrenal vasculature is arranged in such a way that adrenocortical cells are adjacent to blood vessels



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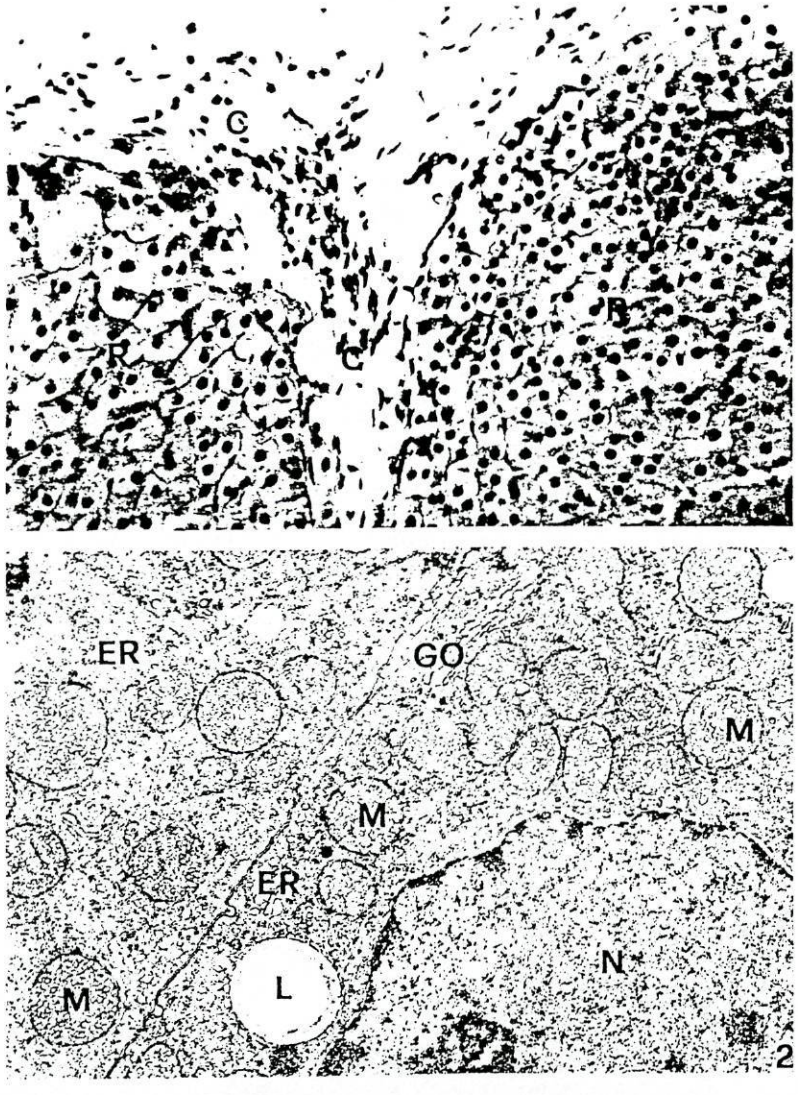
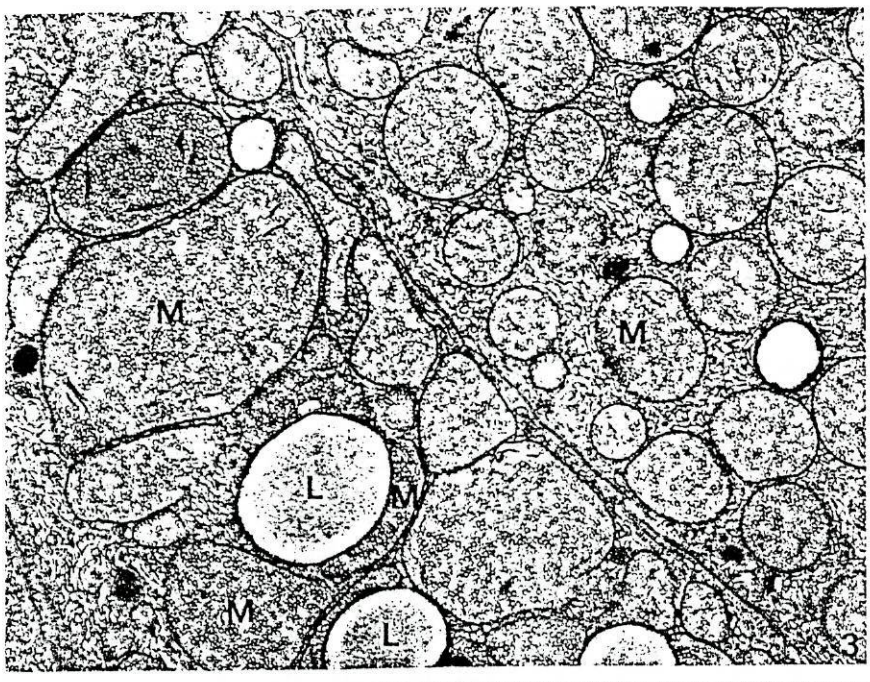
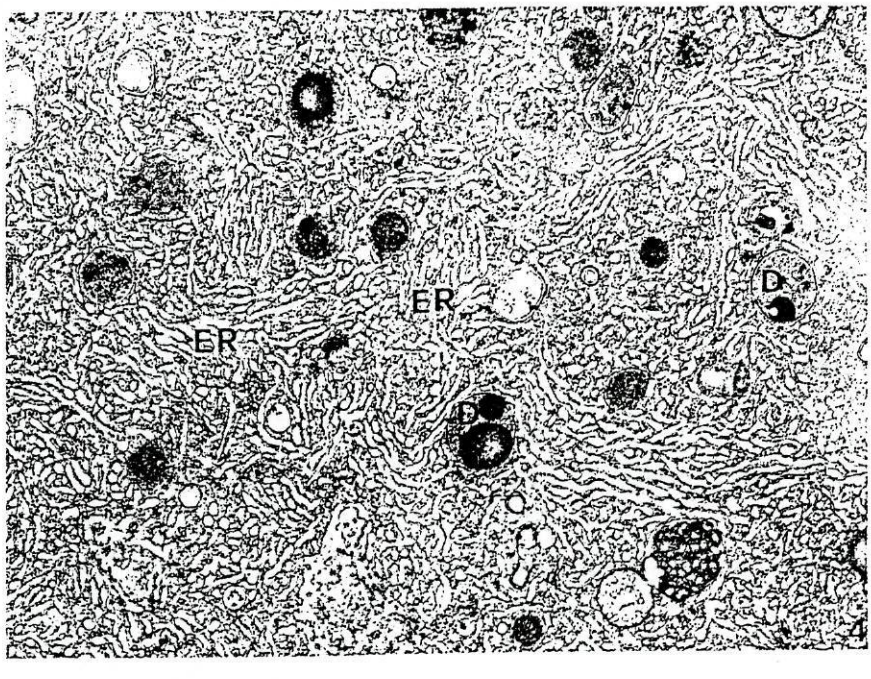



Fig. 1. Light micrograph of adrenal graft. Postautotransplantation = 90 days. Saline-treated animal. R, regenerated cortical tissue; C, connective tissue. ×275.

Fig. 2. Postautotransplantation = 90 days. Saline-treated animal. Adrenal glandular cells show the usual features of steroid-secreting cells. N, nucleus; M, mitochondria; L, lipid droplet; ER, smooth endoplasmic reticulum; GO, Golgi complex ×15,000.

(Pudney et al., 1981), and cells of the vascular endothelium are actively secretory, producing a wide range of substances with multiple functional roles (Vane et al., 1990). In particular, endothelin-1 (ET-1), which was first isolated in 1988 by Yanagisawa et al., promotes the stimulation of aldosterone secretion normally secreted by zona glomerulosa and could have a role in the control of this zone (Hinson et al., 1991a). However, ET-1 seems to modulate the secretion of corticosterone after adrenocorticotrofic hormone (ACTH) stimulation, which induced vasodilatation (Hinson et al., 1991b). ET-1 is produced in the vicinity of ET-binding sites, which strongly suggests that ET-1 is a local autocrine or paracrine hormone. Furthermore, ET-1 probably controls the local production of catecholamines and corticosteroids in the adrenal gland (Masaki, 1993). Moreover, circulating amounts of ET-1 are very small (Suzuki et al., 1989), as is the concen-

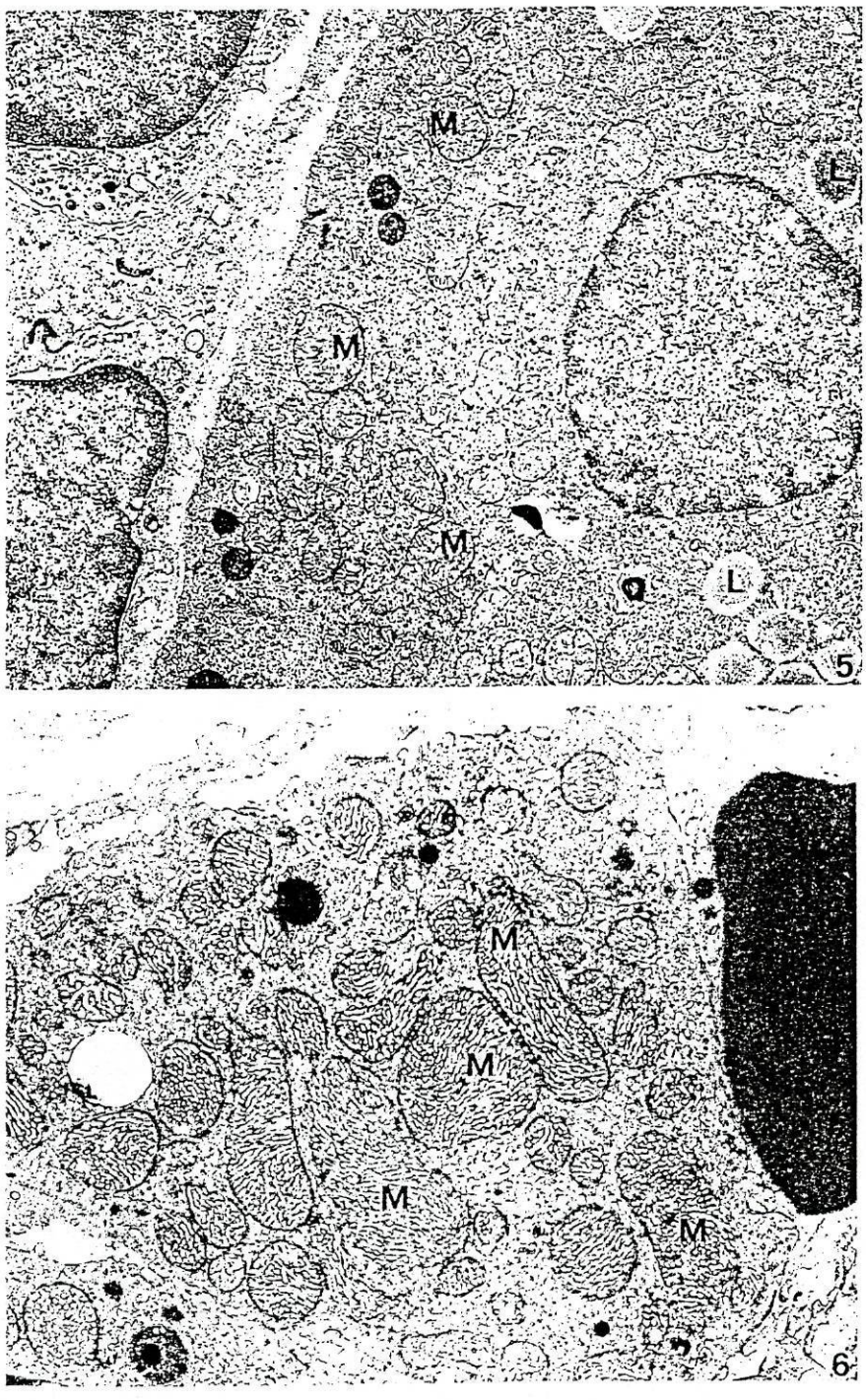



Figs. 3–4. Electron micrographs of autotransplanted adrenal cortex. Postautotransplantation = 90 days. ET-1-treated animals. Graft inner area.

Fig. 3. Fasciculatalike cells showing mitochondria, with atypical organization within the cytoplasm (M). Note mitochondria "wrapping" around lipid droplets (L). ×13,500.

Fig. 4. The same as Figure 3. Numerous dense bodies (D) can be seen, surrounded by cytoplasmic "reticulum lakes" of smooth endoplasmic reticulum (ER). ×20,100.




Figs. 5 and 6 (Legends on following page).

**TABLE 1. Variations of volumetric density (% ± SE) of mitochondria, dense bodies, and smooth endoplasmic reticulum (SER) in ET-1-treated autotransplanted (AT) rats**

| Parameters | Mitochondria | Dense bodies | SER |
|---|---|---|---|
| AT rats | 26.44 ± 3.07 | 1.72 ± 0.46 | 22.76 ± 2.85 |
| $p <$ | 0.001 | 0.0001 | 0.001 |
| AT rats (ET-1) | 40.32 ± 7.22 | 5.10 ± 1.55 | 34.23 ± 6.03 |

tration of receptors, which were previously described in the zona glomerulosa and adrenal medulla of the rat (Davenport et al., 1989; Kohzuki et al., 1991; Belloni et al., 1994). In the present study, we describe the effects of acute ET-1 administration on the morphology and function of rat adrenal autotransplants, a model where the adrenal medulla is absent.

## MATERIAL AND METHODS

Eighteen male Wistar rats from the colony of the Gulbenkian Institute of Sciences (Oeiras, Portugal), with body weights of approximately 200 g, were divided into two experimental groups. Twelve animals (first group) underwent adrenal autotransplantation according to the technique described by Vendeira et al. (1992).

In short, after anesthesia with sodium pentobarbital (0.1 ml/100 g body weight), the animals were bilaterally adrenalectomized. The adrenals were placed in a 0.9% sterile saline solution and cut into small pieces measuring 1–2 mm each. Between 10 and 20 pieces were autotransplanted immediately under the skin of the dorsal region by using the incision previously made. All animals were fed a commercial diet and provided 0.9% saline solution during the first 30 days and subsequently with water until they were killed by decapitation. The rats were housed under normal laboratory conditions with regular diurnal light/dark alterations (12-hr-light, 12-hr-dark cycles). ET-1 (0.5 μg/kg body weight; Sigma Chemical Company, USA) was administered intravenously via the left jugular vein (Renaud, 1969) to each animal 1 hr before death, which took place 90 days after autotransplantation. Animals were handled gently by the same operator to minimize stress. Another six rats (second group) were also autotransplanted and intravenously injected with 0.9% saline solution 1 hr before they were killed. For the assessment of plasma aldosterone and corticosterone and for plasma renin activity, trunk blood was collected and plasma separated by centrifugation and immediately stored at −25°C until assayed. Necropsy was performed on all the animals to search for accessory adrenals. Twelve animals were "sham operated" without removal of their adrenal glands and served as controls. Six normal animals were also included in the experimental procedure for morphological and biochemical controls.

Adrenal grafts and adrenal glands from control animals were fixed in Bouin's liquid for 24 hr and embedded in paraffin. Pieces of adrenal grafts were also fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, at 4°C for 2 hr, postfixed in 1% osmium tetroxide in veronal-acetate buffer, pH 7.3, at 4°C for 2 hr, and Epon embedded. Sections of 1 μm in thickness were stained with methylene blue-azur II for light microscopy (Richardson et al., 1960). Ultrathin sections were stained with alcoholic uranyl acetate (15 min) plus lead citrate (10 min; Reynolds, 1963) and examined in Jeol 100 B and Jeol 100 CX II electron microscopes.

Morphometric studies were performed by using adrenal grafts within 90 days. Three rats for each experiment were used (ET-1 or saline administration). Gold ultrathin sections from three Epon blocks per animal were cut and 10 micrographs per rat were taken at random at 4,500×, so that 30 micrographs were studied for each animal group. A rectangular test—the sides measured 22 and 16 cm and contained 102 lines of constant length (1.3 cm), arranged in 17 equidistant and parallel rows, and the distance between the end points of the lines in every direction was 1.3 cm—was placed on photographic prints that had been enlarged to 13,500× over zones with visible extracellular space. The number of points that fell on the organelles (mitochondria, dense bodies, and smooth endoplasmic reticulum) was used for point-counting volumetry. The number of points ($P_i$) for these structures was transformed into volumetric density in agreement with the formula $V_{vi} = P_i/P_T$ (Weibel, 1973) expressed as a percentage ($P_T$ is the total number of test points). The average values were compared by Student's t test.

For corticosterone assays, the HPLC method (Haughey and Jusko, 1988), with 254-nm absorbance detection, was used. Aldosterone and plasma renin activity were determined by RIA (commercial kits purchased from Sorin Biomedica, Italy). These determinations were done in duplicate. The average values were compared by Student's t test. A $p$ value less than 0.05 was considered significant. To minimize circadian variances, all the animals were killed and trunk blood collected between 2 and 3 p.m.

## RESULTS

### *Light Microscopy*

Adrenal gland structure was identical in normal and sham-operated animals. Concerning the autotransplanted adrenal tissue, no remarkable differences were observed when intravenous ET-1 or saline solution was administered. Briefly, adrenal grafts removed 90 days after the autotransplantation procedure showed a mass of regenerated cortical tissue arranged in nests of glandular cells surrounded or intersected by layers of connective tissue (Fig. 1). Cell arrangement and neovascularization were assembled, disclosing at least two cell populations resembling zona glomerulosa and particularly zona fasciculata. Neovascularization was characteristically more evident in the outer areas. (Fig. 1)

### *Electron Microscopy*

Adrenal gland ultrastructure was identical in normal and sham-operated animals. In addition, in saline-

Figs. 5–8. Electron micrographs of autotransplanted adrenal cortex. Postautotransplantation = 90 days. ET-1-treated animals. Graft outer area. (Figs. 7 and 8 appear on next page.)

Fig. 5. Glomerulosalike cells. M, mitochondria with typical tubular cristae; L, lipid droplets. ×13,500.

Fig. 6. Subcapsular glomerulosalike cells with mitochondria (M) presenting compact and irregular tubular cristae. ×13,500.

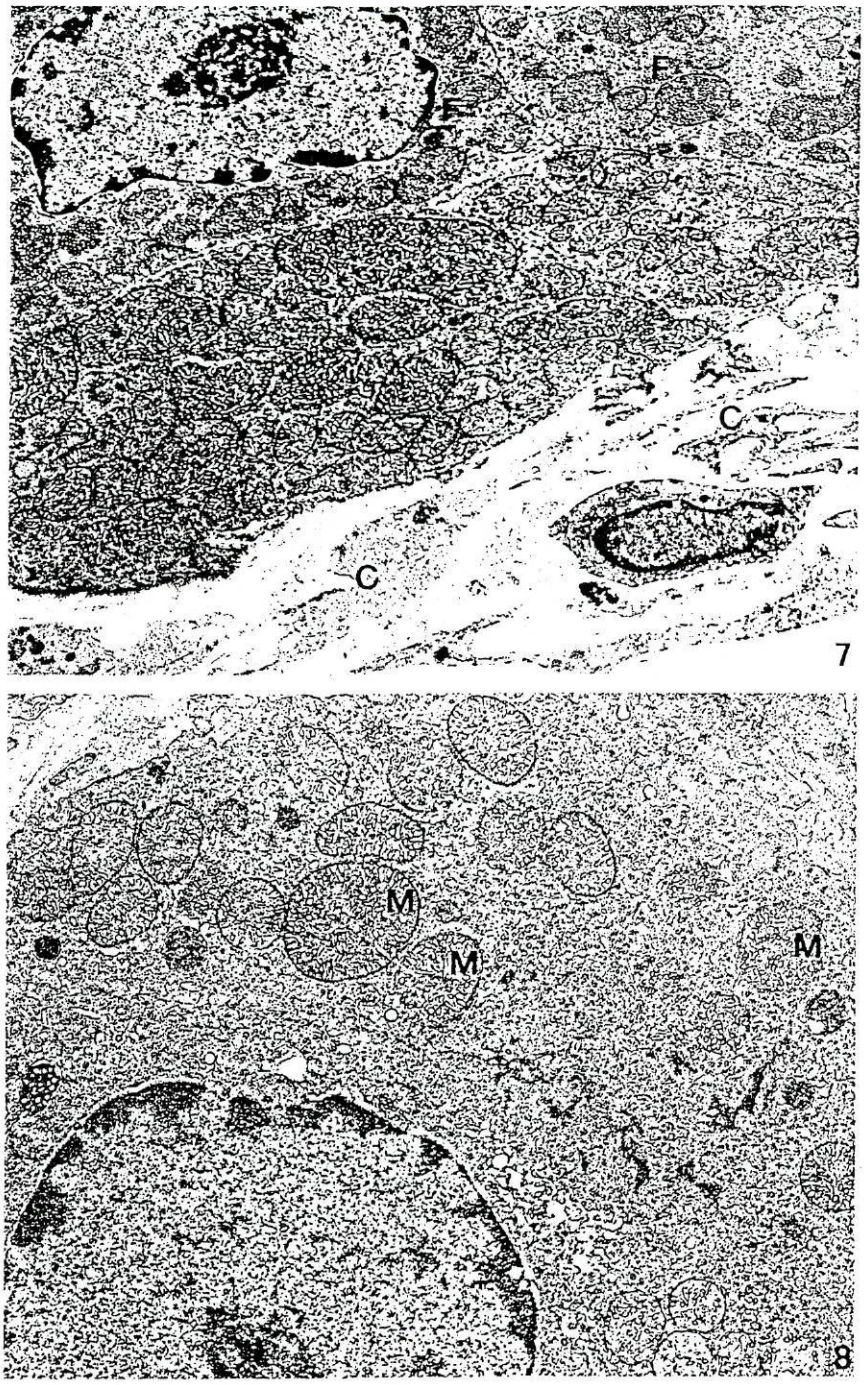



Fig. 7. Interposition with fasciculatalike cells (F) in intimate contact with the connective tissue (C). ×10,200.

Fig. 8. "Hybrid" cell with mitochondria (M) presenting tubulovesicular cristae. Note the absence of lipid droplets. ×13,500.

TABLE 2. Endocrine effects of ET-1 in autotransplanted (AT) rats (mean ± SEM)[1]

| Parameters | Normal rats | Sham-operated rats | AT rats | AT rats (ET-1) |
|---|---|---|---|---|
| Plasma aldosterone, pg/ml (RIA) | 290.5 ± 59.18[a] | 255.83 ± 54.51[d] | 78.25 ± 11.7[g] | 223.12 ± 28.37[j] |
| Serum corticosterone, μg/ml (HPLC) | 0.19 ± 0.02[b] | 0.22 ± 0.03[e] | 0.08 ± 0.004[h] | 0.14 ± 0.011[k] |
| Plasma renin activity, ng/ml/hr (RIA) | 22.79 ± 3.54[c] | 26.45 ± 4.91[f] | 25.37 ± 1.32[i] | 20.47 ± 3.44[l] |

[1]Statistical comparison of the data ($p <$): a vs. d, n.s.; b vs. e, n.s.; c vs. f, n.s.; d vs. g, 0.01; e vs. h, 0.005; f vs. i, n.s.; g vs. j, 0.001; h vs. k, 0.001; i vs. l, n.s.

treated autotransplanted rats, no significant morphological changes were observed. As previously described (Belloni et al., 1990; Vendeira et al., 1992), glandular elements showed the characteristic features of steroid-secreting cells, with mitochondria of tubular and vesicular cristae, abundant profiles of smooth endoplasmic reticulum, and lipid droplets (Fig. 2). "Hybrid" cells resembling those found in the normal zona reticularis, with mitochondria of tubulovesicular cristae, were occasionally observed; however, other characteristic features of these cells such as lipofuscin and apoptotic figures were lacking.

After ET-1 administration, a remarkable difference in glandular ultrastructure was seen. The inner area of the graft showed cells almost invariably like fasciculata; cytoplasmic changes, particularly concerning mitochondria and the smooth endoplasmic reticulum, were often observed, with mitochondria of vesicular type and a high variation in number, size, and arrangement (elongated or in crescent; Fig. 3). Profiles of smooth endoplasmic reticulum sometimes exhibited evidence of hypertrophy, occupying almost all of the cytoplasm and forming "reticulum lakes" (Fig. 4). Numerous dense bodies were occasionally seen in these areas, and lipid droplets could often be observed in close approximation to mitochondria. The outer area of the graft revealed a different glandular pattern, with blood vessels containing a large number of erythrocytes in their lumina. Concerning the subcapsular environment, glandular cells were almost invariably like glomerulosa cells, having the typical mitochondria with tubular cristae (Fig. 5), and were observed in intimate contact with the connective tissue. Some showed a high mitochondrial concentration, with a particular arrangement of their cristae (Fig. 6). Interposed was an occasional fasciculatalike cell (Fig. 7). A distinct type of cell with mitochondria resembling those of the zona reticularis but lacking all the other typical cytological characteristics of reticularis cells (lipofuscin and apoptotic figures; Fig. 8) was also observed in the subcapsular environment; this cell, referred to above as "hybrid," rarely occurred in saline-treated autotransplanted animals.

Quantification of the relative volume of mitochondria, dense bodies, and smooth endoplasmic reticulum showed a significant increase of the volumetric density of all these organelles after ET-1 administration (Table 1).

### *Blood Hormonal Concentrations*

Endocrine data on normal and sham-operated rats are shown in Table 2. No significant changes could be seen between these two groups of animals. Autotransplanted rats showed a significant decrease in aldosterone and corticosterone plasma concentrations. However, plasma renin activity underwent an insignificant decrease. The acute intravenous administration of ET-1 to rats previously autotransplanted produced a significant rise in corticosterone (0.14 μg/ml vs. 0.08 μg/ml) and particularly aldosterone (223 pg/ml vs. 78 pg/ml) concentrations. The plasma renin activity (20.47 ng/ml/hr vs. 25.37 ng/ml/hr) was not significantly changed (Table 2).

## DISCUSSION

Adrenal gland autotransplantation provides a very interesting model of adrenal regeneration, where adrenocortical cells proliferate and undergo functional and morphological differentiation without the control of the zona medullaris (Vendeira et al., 1992). As seen in most vertebrates, steroid-secreting and chromaffin tissue in the adrenal gland are located together in spite of their different embryological origin, and it is now evident that cortex and medulla are functionally interlinked (Carballeira and Fishman, 1980; Hinson, 1990).

In 1987, Holzwarth et al. suggested that adrenal neuromodulation could be regulated by splanchnic nerve activity, and the release of vasoactive intestinal peptide (VIP) by splanchnic stimulation has been demonstrated (Bloom et al., 1987; Kong et al., 1989). In addition, Gallo-Payet et al. (1987) and Bornstein et al. (1994) referred to the relevant paracrine mechanisms within the adrenal cortex, and several neuropeptides were identified in the gland (Malendowicz, 1993). Concerning adrenal growth, VIP and neuropeptide Y chronically stimulate the growth of zona glomerulosa and aldosterone secretion (Mazzocchi et al., 1987; Rebuffat et al., 1988), but such an effect was observed only when the architecture of the zona glomerulosa was preserved. The effect from VIP seemed to be mediated by local release of adrenaline (Hinson et al., 1992). However, neural connections seem to be implicated in compensatory adrenal growth (Dallman et al., 1976). However, all studies were performed on intact animals.

The adrenal gland autotransplantation technique provides a model to study adrenal growth in the absence of chromaffin tissue or a nerve supply. Previously, by using autoradiographic techniques, we assumed that adrenal regeneration in the autotransplant process took place at the graft periphery and proceeded from subcapsular glomerulosa cells (Vendeira et al., 1992). In addition, autoradiographic studies using the gland enucleation model showed a rapid proliferation before the end of the first month, with mitotic cells located in a few cell layers of the subcapsular region (Seki et al., 1969). This result suggested a preponderant role for the classical adrenoglomerulotrophic factors (ACTH, angiotensin II, and K+) in stimulating the mitotic activity of zona glomerulosa (Nussdorfer,

1986). Moreover, when ET-1 is chronically administered, it is able to enhance specifically the growth and steroidogenic capacity of rat zona glomerulosa and exert a strong proliferogenic effect on zona glomerulosa (Mazzocchi et al., 1990b). Our results give some support to the understanding of ET-1 effects in adrenal modulation. In fact, the acute stimulation with this peptide enhanced aldosterone secretion and induced cytoplasmic changes in glomerulosalike cells, which could not be reached in intact animals by Mazzocchi et al. (1990a). However, in our experiments, no significant variations concerning plasma renin activity were observed, which reinforce the possibility that ET-1 acts directly on zona glomerulosa. Paradoxically, the acute administration of angiotensin II to autotransplanted rats does elicit an insignificant increase in aldosterone plasma concentrations (Belloni et al., 1990), which markedly increased when the animals where chronically sodium restricted (Belloni et al., 1991). These data suggest that adrenal-regenerated glandular cells might be, at least in acute experiments, more responsive to ET-1 than to angiotensin II. ET-1 seems to share some common functional aspects with neuropeptides, which have been clearly evident in the capsule and zona glomerulosa (Hinson et al., 1994). However, according to our present knowledge, ET-1 has not yet been identified in adrenal nerve terminals. Hence, immunohistochemical studies using antibodies against ET-1 and studies with chronic stimulation of autotransplanted adrenals with both ET-1 and angiotensin II will be necessary to clarify these effects.

Concerning glucocorticoid levels, we noticed that serum corticosterone concentrations were 50% lower in autotransplanted rats than in sham-operated animals. These differences might be due to a relatively low mass of adrenocortical tissue in the autotransplanted animals because ACTH levels are chronically increased (Wilkinson et al., 1981; Engeland, 1984; Belloni et al., 1990). Other factors are important in adrenal regeneration such as the pituitary-generated N-terminal proopiomelanocortin (N-POMC) peptide (Estivariz et al, 1988). In addition, Thorne et al. (1991) demonstrated that POMC was synthesized in the adrenal medulla and thus might influence corticosteroid biosynthesis in a paracrine way (Szalay, 1993); however, in our model the medulla is absent. Recent data on growth factors in the adrenal support the view that have fibroflant growth factor (bFGF) has a compensatory adrenal growth response (Basile and Holzwarth, 1994). Ho and Vinson (1994) referred to an autocrine/paracrine mitogenic action of bFGF and insulin-like growth factor-1 (IGF-1). Nevertheless, it seemed intriguing that the acute administration of ET-1 to autotransplanted animals led to a significant increase in corticosterone blood levels, all the more because ET-1 receptors are located at zona glomerulosa. The potential role of ET-1 on increasing the blood flow through the graft should be considered. A low concentration of ET-1 elicits vasodilatation rather than vasoconstriction (Masaki, 1993). However, Hinson et al. (1991b), using the in situ isolated perfused rat adrenal gland, demonstrated that the administration of ACTH caused an increase in the rate of immunoreactive endothelin secretion. They concluded that endothelin may mediate the effect of ACTH-induced vasodilatation on corticosterone secretion. Therefore, our results might reflect the effect of a direct stimulation (ET-1 receptors) of a "hybrid" cell pool located between the two outer and inner zonae, irreversibly directed to fasciculata differentiation. These cells seem like reticularis cells; however, they disclose neither the "aging pigment" lipofuscin nor apoptotic figures usual in reticularis cells (Penney et al., 1963; Rhodin, 1971; Wyllie et al., 1980). In the same way, the increased number of glomerulosalike cells (with or without stimulation features) reflect the stimulation of these cells, which can present ET-1 receptors.

A cell layer located between the zona glomerulosa and fasciculata and named the zona intermedia has been reported (Cater and Lever, 1954; Neville and O' Hare, 1982). The layer consisted of three to five layers of cells with ultrastructural features that were identical to our "hybrid" cells. These researchers have suggested that these are transitional cells between the zona glomerulosa and zona fasciculata. Recently, within the same location (Mitani et al., 1994), a novel cell layer, without corticosteroid-synthesizing enzymes, was found in the rat adrenal and considered to be the progenitor layer of the cells of the adrenal cortex zonae. However, the fact that autotransplantation techniques provoke an almost total necrosis of the parenchymal cells, with the exception of the capsule and a few subcapsular cells, excludes the hypothesis that such a zone may influence the growth of adrenal differentiated cells. Moreover, zona glomerulosa cells in culture first secrete aldosterone and then corticosterone and the cells later acquire the ultrastructural features of fasciculata/reticularis cells (Hornsby et al., 1974).

In summary, it is unquestionable that ET-1 in the present study leads to adrenal morphological and biochemical alterations, suggesting a role in the modulation of steroidogenesis. Further studies, namely immunohistochemical ones are required, to obtain the definition of autotransplanted adrenal zonation in the absence of adrenal medulla or neural connections.

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# PUBLICAÇÃO III

*New insights into zonal differentiation of adrenal autotransplants in the rat: an immunohistochemical study*

# New insights into zonal differentiation of adrenal autotransplants in the rat: an immunohistochemical study

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## Abstract

Adrenal gland autotransplantation, an interesting model of adrenal regeneration, provides the reconstruction of distinct functional and morphological zonae. An immunohistochemical study of the adrenal gland of adult male rats after autotransplantation and endothelin-1 (ET-1) stimulation was carried out. The technique involved total adrenalectomy and immediate autotransplantation of small adrenal pieces under the skin of the dorsal region. The animals were killed 90 days after the autotransplantation and 1 h after intravenous ET-1 administration. Sections of recovered adrenal grafts were incubated with IZAb, a monoclonal antibody which interacts with an antigen (IZAg) predominantly found in rat adrenal inner zones. Saline-treated control autotransplanted animals showed IZAb immunostaining in almost all adrenocortical tissue, with the exception of small clusters of cells beneath the capsule. ET-1-treated animals exhibited an extended zone devoid of immunostaining and located in the subcapsular area. In addition, ET-1-stimulated animals showed significant increases in aldosterone as well as corticosterone concentrations in plasma. These results revealed that ET-1 stimulated the development of an extended subcapsular zone lacking IZAg expression, an effect that suggests its role in zona glomerulosa induction in these animals.

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## Introduction

The data concerning mammalian differentiation, namely adrenocortical zonation, remain unclear at present. There are two main theories: the cell migration and the zonal theories. According to the cell migration theory (da Costa 1951, Wright *et al.* 1973, Nussdorfer 1986), the adrenocortical cells arise in zona glomerulosa, migrate into zona fasciculata and die in zona reticularis. Zajicek *et al.* (1986) suggested that cell migration is accompanied by changes in the morphology of adrenal cells which do not necessarily reflect their functional specificity (Ganguly 1991). In a previous study (Vendeira *et al.* 1996), we used the rat adrenal autotransplantation technique as a model of adrenal regeneration, and we have described the morphological and biochemical changes resulting from acute stimulation with endothelin-1 (ET-1), a 21 amino acid peptide first isolated by Yanagisawa *et al.* (1988). ET-1 has been shown to have a role in cellular differentiation and function (Simonson & Dunn 1991, Takuwa 1993) and to stimulate aldosterone secretion in rats (Mazzocchi *et al.* 1990*a*,*b*, Hinson *et al.* 1991*a*,*b*). In addition, its receptors are present in the adrenal zona glomerulosa and medulla (Davenport *et al.* 1989, Koseki *et al.* 1989, Kohzuki *et al.* 1991, Belloni *et al.* 1994). In Vendeira *et al.* (1996), regenerated adrenal tissue exhibited some morphological zonation; however, such zonation was not the classical one observed in *in situ* glands. It was even dificult to identify adrenocortical cells located beneath the capsule or connective tissue septa; the remaining cell population was similar in arrangement to the zona fasciculata-like cells in the intact animal. ET-1, when studied by conventional light microscopy, did not produce appreciable alterations, in spite of the increase in plasma aldosterone as well as corticosterone concentrations.

This led us to create a more discriminatory method for the analysis of the finest cellular alterations. In this study, we have used a monoclonal antibody (IZAb) which interacts with an antigen found in rat adrenal inner zones (zona fasciculata and zona reticularis) and not in the glomerulosa zone (Laird *et al.* 1988, Barker *et al.* 1992, Ho *et al.* 1994). This antibody recognizes a 60 kDa protein (IZAg-2) which is processed to yield a smaller protein (IZAg-1, 30 kDa) after adrenocorticotrophin (ACTH) stimulation. Since ACTH plasma levels are high during autotransplantation of adrenal tissue (Wilkinson *et al.* 1981, Engeland 1984, Belloni *et al.* 1990), we studied IZAg expression in control conditions and after ET-1 administration.

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**Figure 1** Normal adult rat (non-autotransplanted). IZAb immunostaining (1:50); avidin/biotin complex technique. C, capsule; ZG, zona glomerulosa; IN, zona fasciculata+zona reticularis (inner zone); M, medullary cells. Scale bar=100 μm.

## Materials and Methods

Wistar rats, of approximately 200 g body weight, were obtained from the colony of the Gulbenkian Institute of Sciences, Oeiras, Portugal. They were divided into two experimental groups. All animals underwent bilateral adrenalectomy and adrenal autotransplantation, following our previously described technique (Vendeira *et al.* 1992). The animals were fed on a commercial diet and provided with 0·9% saline to drink during the first 30 days following surgery, and subsequently with water. They were housed under normal laboratory conditions with regular diurnal light:darkness conditions.

Animals were used 90 days after autotransplantation. In the first experimental group (six animals), ET-1 (0·5 μg/kg body weight; obtained from Sigma Chemical Co., St Louis, MO, USA) was administered intravenously via the left jugular vein (Renaud 1969) and under anaesthesia with sodium pentobarbital, 1 h before they were killed by decapitation. At the same time, a control group (three rats)

**Figure 2** (*a*) Post-autotransplantation (90 days) in a saline-treated animal. IZAb immunostaining (1:50) and the avidin/biotin complex technique are common to (*a–d*); C, capsule; R, regenerated adrenal tissue. (*b*) As (*a*); note adrenal glandular cells devoid of immunostaining (arrows) beneath the capsule; ZF, inner area (fasciculata-like cells). (*c*) Post-autotransplantation (90 days) in an ET-1-treated animal; SC, subcapsular zone. (*d*) As (*c*); note the similarity with intact adrenal glands (Fig. 1), except for width variations. Scale bars=10 μm.

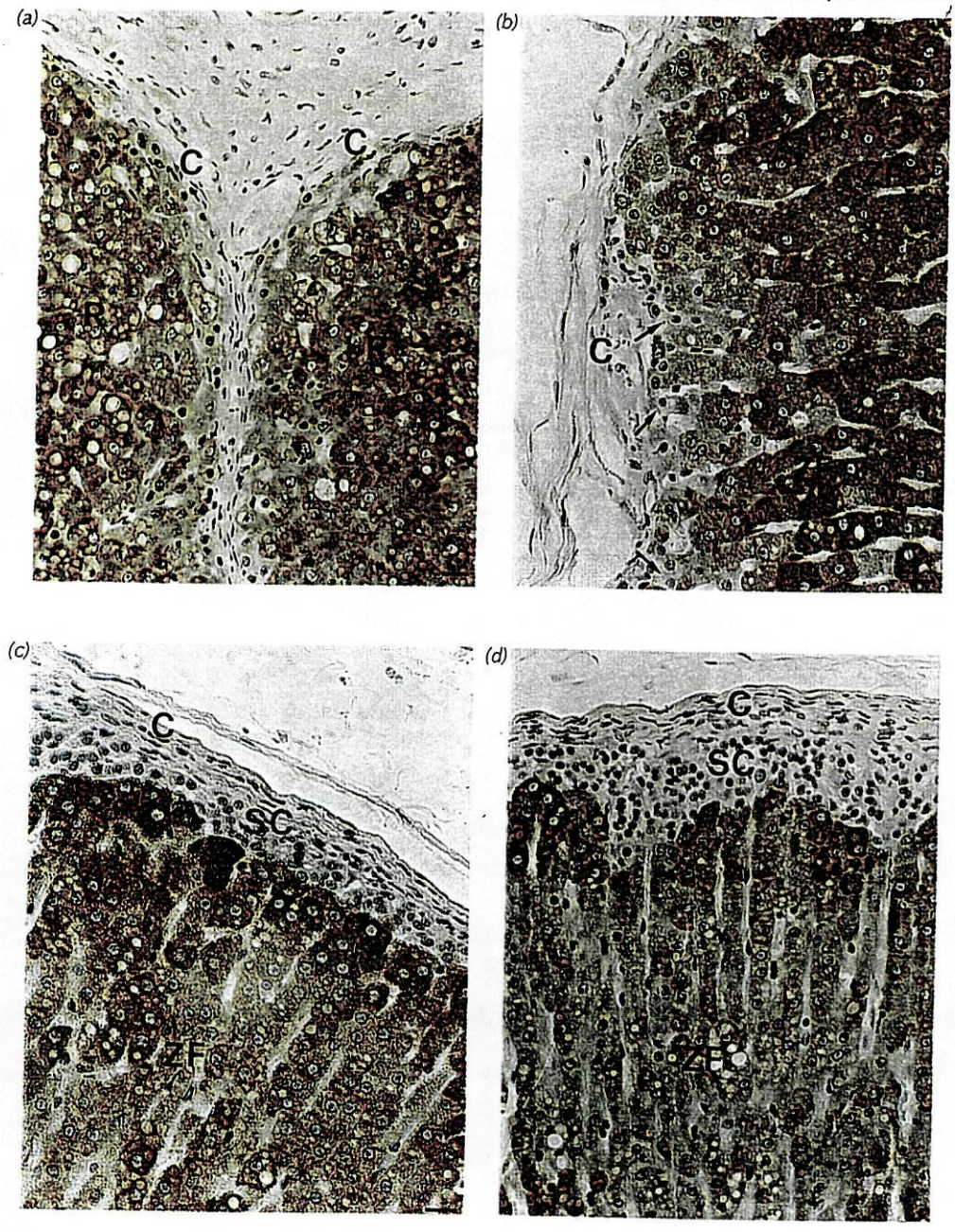
(a)
C
C
R
R
(b)
C
(c)
C
SC
ZF
(d)
C
SC
ZF

**Table 1** Endocrine effects of ET-1 in autotransplanted (AT) rats. The data presented are the means ± S.E.M.

| | AT rats | *P* values | AT rats (ET-1) |
|---|---|---|---|
| Plasma aldosterone (pg/ml) (RIA) | 70·16 ± 13·91 | <0·001 | 234·25 ± 30·47 |
| Serum corticosterone (μg/ml) (HPLC) | 0·07 ± 0·007 | <0·001 | 0·14 ± 0·012 |
| Plasma renin activity (ng/ml per h) (RIA) | 24·79 ± 1·67 | NS | 21·57 ± 3·78 |

NS, not significant.

was similarly injected intravenously with 0·9% saline solution. A group of four normal animals (non-autotransplanted) was also used to define normal IZAg expression. Animals were handled gently by the same operator to minimize any stress response. Trunk blood was collected for plasma aldosterone, corticosterone and renin activity assays, and the plasma was separated by centrifugation and immediately stored at − 25 °C until required for assay.

All animals were autopsied immediately to ascertain the presence of natural accessory adrenals. If present, the animals were removed from the experimental groups. Adrenal grafts were removed and immediately fixed in 4% formaldehyde in PBS (pH 7·4, 0·1 mol/l) for 18 h at 4 °C. Fixed adrenal tissue was dehydrated and then embedded in paraffin wax. Sections (5 μm) were cut and mounted on gelatine-coated glass slides. After dewaxing, sections were washed in Tris–buffered saline (pH 7·5). Sections were then incubated for 30 min with IZAb (1:50), followed by biotinylated rabbit anti-mouse IgG and peroxidase-conjugated avidin (avidin–biotin complex; DAKO Ltd, Copenhagen, Denmark). Visualization of the peroxidase activity was achieved by incubation for 20 min with 3,3′-diaminobenzidine (Sigma) and $H_2O_2$. Sections were counterstained with haematoxylin. Washed sections were then mounted with Entellan (Merck, Darmstadt, Germany), and viewed under a light microscope. To establish the specificity of the above immunochemical staining, sections were also incubated with mouse IgG 1 negative control serum (Dakopatts A/S Produktionsvej, Glostrup, Denmark), in place of the specific antibody described above. No specific immunohistostaining of IZAg was observed under these experimental conditions. Twelve tissue sections from six-ET-1 treated animals and nine sections from three saline-treated animals were used for counting the number of glomerulosa-like cell layers.

We used HPLC for corticosterone assays (Haughey & Jusko 1988) with absorbance detection at 254 nm. Aldosterone and plasma renin activity were determined by RIA (commercial kits purchased from Sorin Biomedica, Italy). In order to minimize circadian variation, all animals were killed and their trunk blood was collected between 1400 and 1500 h.

## Results

In normal rats (non-autotransplanted), specific IZAb binding was confined to the inner adrenocortical zones (zona fasciculata+zona reticularis). The zona glomerulosa and medulla were unstained, except for a few isolated cortical cells within the medulla (Fig. 1).

In autotransplanted animals injected with saline solution, immunostaining with IZAb was observed throughout almost all of the regenerated adrenocortical tissue (Fig. 2*a*), with the exception of some dispersed small clusters of cells beneath the capsular tissue or adjacent connective tissue septa (Fig. 2*b*). These cells, identified as glomerulosa-like cells could sometimes define a one cell-thick layer. Medullary tissue did not survive the autotransplantation procedure.

Following ET-1 administration, all adrenal grafts showed the presence of an extended subcapsular zone devoid of immunostaining. The bulk of the regenerated tissue, apparently corresponding to adrenocortical inner zones, was IZAb-positive (Fig. 2*c*). The unstained cell layers, about three to eight cells thick (Fig. 2*d*), were very similar in appearance to the zona glomerulosa of intact adrenal glands (compare with Fig. 1), although variations in the width of this zone were frequent.

Acute intravenous ET-1 administration produced a significant rise in aldosterone plasma concentration (234 vs 70 pg/ml). The plasma concentration of corticosterone also showed a significant rise (0·14 vs 0·07 mg/ml) in autotransplanted rats, when compared with saline-treated controls. Plasma renin activity (21·6 vs 24·8 ng/ml per h) was not significantly affected (Table 1).

## Discussion

Since IZAb recognizes an inner zone-specific factor, it may be used to monitor adrenocortical zonation (Laird *et al.* 1988). In fact, it will permit a clear distinction between outer and inner zonae. In the intact gland, IZAg expression has proved to be a good marker for unequivocal identification of adrenal cell types (Ho & Vinson 1993).

In the present study, IZAb defined both outer and inner adrenocortical areas in autotransplanted adrenal glands.

However, the outermost zone was restricted to small clusters of unstained cells located in the subcapsular region. After ET-1 administration, an apparent extension of the subcapsular zone lacking IZAg expression was observed. This effect suggests a regulatory mechanism of ET-1 in the structure and function of zona glomerulosa, which is supported by the significant increase of aldosterone plasma concentrations in autotransplanted ET-1-treated animals. In turn, ET-1 can stimulate the conversion of angiotensin I to angiotensin II and potentiate the angiotensin II-induced production of aldosterone (Cozza *et al.* 1992, Levin 1995). The measurement of plasma angiotensin II levels seems to be important in order to define the possible combined role of ET-1 in the renin-angiotensin-aldosterone system. However, endothelins have been shown to act directly on adrenal glomerulosa cells, causing stimulation of aldosterone secretion (Woodcock *et al.* 1990). In fact, the IZAg dynamic changes suggest that some direct effect of ET-1 accounts for this occurrence.

The distribution of IZAg in the zona fasciculata is similar to that of 11β-hydroxylase, as shown by an *in situ* hybridization technique (Ho & Vinson 1993). Since aldosterone synthase can catalyse all of the steroidogenic steps in zona glomerulosa from deoxycorticosterone to aldosterone, it has been considered that 11β-hydroxylase is not essential for aldosterone biosynthesis (Lauber & Müller 1989, Ogishima *et al.* 1992). However, after chronic ACTH treatment the distribution of 11β-hydroxylase mRNA and IZAg both clearly extend towards the subcapsular region (Ho & Vinson 1993), and aldosterone secretion is inhibited. Since, in our model, plasma ACTH concentrations remain high during adrenocortical regeneration, it seems plausible that they reflect the ACTH condition of the intact animals. On the other hand, after ET-1 treatment the effect of ACTH is counteracted, IZAg expression disappears in the outermost layers, and aldosterone output is enhanced.

The mechanism involved in the acute stimulatory effect of ET-1 is unknown. According to Mazzocchi *et al.* (1990*b*), after chronic infusion with ET-1, this regulatory effect involves the stimulation of *de novo* synthesis, both of the steroidogenic enzymes and of the membrane framework in which they are located. However, it is unlikely that synthesis of a new aldosterone synthase could be sufficient to account for this change with this time of exposure. The enhanced availability of 18-hydroxydeoxycorticosterone precursor, a pool usually confined to the inner adrenocortical zone, could account for this acute stimulatory action, as in other situations (Vinson *et al.* 1995). On the other hand, the elimination of IZAg expression is intriguing, suggesting that this acute process is, in some way, important for the secretory response of aldosterone.

Recently, a new cell layer located between the outer and inner areas was described in the rat adrenal cortex (Mitani *et al.* 1994). Such cells exhibited no corticosteroid-synthesizing enzymes, namely aldosterone synthase and 11β-hydroxylase. This layer seems to represent a transitional state between glomerulosa and fasciculata zonae (Cater & Lever 1954, Neville & O'Hare 1982, Gomez-Sanchez *et al.* 1988), and, as a dynamic area, it is probably more sensitive to local regulators. The increased corticosterone plasma concentration in ET-1-treated autotransplanted animals, also observed in the *in situ* perfused rat adrenal gland preparation where an increased secretion of immunoreactive ET-1 into the adrenal vein was observed after ACTH perfusion (Hinson *et al.* 1991*a*), could be due to the stimulation of these intermediate cells, indicating a redifferentiation to fasciculata-type cells. However, the time of exposure to ET-1 in the present study seems to be very short for this to occur. Further studies will be useful in order to clarify the role of this cell layer in terms of steroidogenic enzyme expression and interactions between angiotensin II, aldosterone and ET-1.

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# PUBLICAÇÃO IV

*Effects of prolonged infusion of basic fibroblast growth factor and IGF-I on adrenocortical differentiation in the autotransplanted adrenal: an immunohistochemical study*

# Effects of prolonged infusion of basic fibroblast growth factor and IGF-I on adrenocortical differentiation in the autotransplanted adrenal: an immunohistochemical study

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**Abstract**

Adrenocortical regeneration after adrenal autotransplantation provides a model for the study of local autocrine/paracrine mechanisms involved in the growth and differentiation of the adrenal cortex. To study the possible involvement of some growth factors, namely basic fibroblast growth factor (bFGF, FGF-2) and insulin-like growth factor I (IGF-I), in cell differentiation, immunohistochemical and ultrastructural studies were carried out on adrenal autotransplants in adult male rats. To distinguish between fasciculata and glomerulosa-like cells with accuracy, tissue sections were immunostained with IZAb, which recognizes the inner zone antigen (IZAg) present in fasciculata and reticularis cells but absent from the glomerulosa, and by electron microscopy. IGF-I-treated animals exhibited a clear glomerulosa-like zone that was devoid of IZAb immunostaining. In this outer subcapsular area, ultrastructural examination showed cells containing mitochondria with irregular cristae resembling those of the fetal or immature glomerulosa cells. In contrast, no significant morphological differences were observed in bFGF-treated animals when compared with those from saline-treated controls, in both of which, IZAb immunostaining occurred in almost all adrenocortical cells, with no clear zonation or glomerulosa, as seen in the intact animal. Plasma aldosterone and corticosterone concentrations were lower in autotransplanted control animals than in intact controls, although plasma renin activities were similar. IGF-I treatment significantly increased aldosterone concentrations, whereas corticosterone and plasma renin activity were reduced. bFGF infusion further reduced plasma aldosterone, although plasma renin activity and corticosterone were unaffected. These results suggest that the two growth factors have different effects on zonal differentiation and function in the autotransplanted gland. In particular, bFGF, by reducing glomerulosa function, appears partly to replicate the actions of ACTH in normal animals. In contrast, IGF-I enhances the glomerulosa secreting phenotype and diminishes that of the fasciculata/reticularis, possibly replicating the actions of angiotensin II or a low sodium diet.

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## Introduction

Adrenal gland autotransplantation provides a useful model to study adrenocortical regeneration in laboratory animals (Ingle & Higgins 1938, Belloni *et al.* 1990). This is particularly so as morphological zonation may also be reproduced (Saxe & Connors 1985, Vendeira *et al.* 1992).

Transplanted tissue may recover its functions, at least partially, but normally not reflecting morphological zonation, as neither glucocorticoid nor mineralocorticoid secretion is fully restored; this has been demonstrated in both humans (Prinz *et al.* 1989, Demeter *et al.* 1990, Lucon *et al.* 1993) and rodents (Belloni *et al.* 1991, Ganguly 1991, Sarria *et al.* 1995, Vendeira *et al.* 1996*a*).

As we have previously demonstrated (Vendeira *et al.* 1992), regeneration processes in the autotransplanted gland start at the periphery of the graft, proceeding from subcapsular glomerulosa-like cells. A partial adrenocortical zonation, with differentiated inner and outer areas, was observed at day 90 after autotransplantation, even in the absence of the medulla (Vendeira *et al.* 1996*b*). Conceivably, this may be enhanced by administration of substances that specifically stimulate the growth and steroidogenic capacity of the zona glomerulosa, perhaps including the wide range of neuropeptides that have been identified in

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the capsule and zona glomerulosa of adrenal glands (Hinson *et al.* 1992, 1994, Malendowicz 1993, Nussdorfer 1996). Vascular products may also be involved, for example endothelin-1, which can both stimulate proliferation of the rat adrenal zona glomerulosa (Mazzocchi *et al.* 1992, 1997, Belloni *et al.* 1996) and potentiate aldosterone secretion (Cozza & Gomez-Sanchez 1990, Mazzocchi *et al.* 1990*a*, *b*, Hinson *et al.* 1991, Nussdorfer *et al.* 1997). Indeed, previous studies have emphasized a role for endothelin-1 in adrenals regenerating after autotransplantation (Vendeira *et al.* 1996*a*) or adrenal enucleation (Malendowicz *et al.* 1997).

Some growth factors are also known to be important. Much evidence supports the modulatory role of basic fibroblast growth factor (bFGF, FGF-2) and insulin-like growth factor-I (IGF-I) in the growth and regulation of a wide array of biological systems (Goustin *et al.* 1986, Han *et al.* 1987, Roith 1997, Bikfalvi *et al.* 1997, Ray & Melmed 1997). More specifically, in the context of steroidogenic organs, IGF-I is thought to induce and maintain differentiated functions of Leydig, ovarian granulosa and adrenocortical cells (Bergh *et al.* 1991, Penhoat *et al.* 1994). In the adrenal, this concept is strengthened by the fact that adrenocortical cells secrete IGF-I during proliferation induced by enucleation or unilateral adrenalectomy, or by adrenocorticotrophin (ACTH) or angiotensin II treatment (Penhoat *et al.* 1989, Jackson *et al.* 1991), and that IGF-I promotes both mitosis and steroidogenesis in human, bovine and rat adrenal cortex (Mesiano *et al.* 1993, Viard *et al.* 1993, Penhoat *et al.* 1994, Weber *et al.* 1997). Changes in translation of mRNA coding for both bFGF and IGF-I support the view that growth and differentiation of the rat adrenal cortex may at least partly be mediated by these factors in an autocrine/paracrine manner (Mesiano *et al.* 1991, Ho & Vinson 1995). bFGF is also a potent mitogen of bovine adrenocortical cells and rat capsule glomerulosa *in vitro* (Gospodarowicz *et al.* 1977, Basile & Holzwarth 1993). In addition, after unilateral adrenalectomy, bFGF was localized immunohistochemically in cells of the rat adrenal zona glomerulosa and medulla (Basile & Holzwarth 1993, 1994). Taken together, these data support the role of bFGF in autocrine/paracrine stimulation and in the compensatory adrenal growth response.

As we have pointed out (Vendeira *et al.* 1996*b*), a more discriminatory morphological method of analysis is necessary for experimental regeneration studies. In this study, we describe the effects of chronic treatment with bFGF and IGF-I on the biochemistry and morphology of adrenal autotransplants. The specific antibody, inner zone antibody (IZAb), which labels the antigen IZAg, which is found only in fasciculata and reticularis cells of the rat adrenal cortex (Laird *et al.* 1988, Barker *et al.* 1992, Ho *et al.* 1994), was used as a tool for cellular analysis. Electron microscope studies were employed to give some insight into the ultrastructural alterations in the subcapsular glomerulosa-like cells.

## Materials and Methods

Twenty-six male Wistar rats from the colony of the Gulbenkian Institute of Sciences (Oeiras, Portugal), with body weights of approximately 200g, were divided into four experimental groups. After intraperitoneal anaesthesia with sodium pentobarbital, the animals of three of these groups underwent bilateral adrenalectomy and adrenal autotransplantation as previously described (Vendeira *et al.* 1992). Briefly, bilateral adrenalectomy was performed by a subcostal extraperitoneal incision, and the adrenals were placed in a 0·9% sterile saline solution and cut into small pieces measuring 2 mm each including the capsule. All the fragments were immediately autotransplanted under the skin of the dorsal region. All animals were fed on a commercial diet and provided with 0·9% saline solution during the first 30 days after surgery, and subsequently with water until they were killed by decapitation. The rats were housed under normal laboratory conditions with regular diurnal light/dark alterations (12 h light/12 h darkness cycles). Seven days before being killed (at 90 days after the autotransplantation), the animals were chronically infused with either 0·9% saline solution (controls, five animals) or 0·9% saline containing bFGF (eight animals) or IGF-I (eight animals) (Bachem Feinchemikalien, Bubendorf, Schwitzerland). The delivery of the drugs was by intraperitoneal infusion after implantation of Alzet mini-osmotic pumps (model 2001) with a reservoir volume of 200 µl (Alza pharmaceuticals, Palo Alto, CA, USA). The rate of delivery was 0·2 µg/kg per h, with a pump rate of 1·0 µl/h. Animals were handled gently by the same operator to minimize stress responses. For the assessment of plasma aldosterone and corticosterone and plasma renin activity, trunk blood was collected. Plasma was separated by centrifugation and immediately stored at -25 °C until assayed. A group of five intact animals was also used to define normal IZAg expression as well as normal plasma steroid concentrations. Necropsy was carried out on all the animals to search for accessory adrenals. Adrenal grafts were removed and fixed in 4% formaldehyde in PBS (pH 7·4, 0·1 mol/l) for 18 h at 4 °C. Fixed adrenal tissue was dehydrated and then embedded in paraffin wax. Sections (5 µm) were cut and mounted on gelatine-coated glass slides. After being dewaxed, sections were washed in Tris-buffered saline (pH 7·5). They were then incubated for 30 min with IZAb (1:50), followed by biotinylated rabbit anti-mouse IgG and peroxidase-conjugated avidin (avidin–biotin complex; Dako Ltd, Copenhagen, Denmark). Visualization of the peroxidase activity was achieved by incubation for 20 min with 3,3′-diaminobenzidine (Sigma) and $H_2O_2$. Sections were counterstained with haematoxylin. Washed sections were then mounted with Entellan (Merck, Darmstadt, Germany) and viewed under a Leitz light microscope. To establish the specificity of the immunohistochemical staining, sections were also incubated with mouse IgG-I-

negative control serum (Dakopatts A/S Produktionsvej, Glostrup, Denmark) in place of the specific antibody described above. Twenty-four grafts from eight bFGF-treated and eight IGF-I-treated animals and nine from five saline-treated animals were used to determine the number of glomerulosa-like cell layers. Pieces of adrenal grafts were also fixed in 2·5% glutaraldehyde in 0·1 M cacodylate buffer (pH 7·2) for 2 h at 4 °C, postfixed in 1% osmium tetroxide in veronal/acetate buffer (pH 7·2) for 2 h at 4 °C, and Epon embedded. Sections 1 μm thick were stained with methylene blue/azur II for light microscopy (Richardson *et al.* 1960) to identify the adrenal subcapsular and inner zones. Ultrathin sections from these areas were stained with alcoholic uranyl acetate (15 min) plus lead citrate (10 min) (Reynolds 1963) and examined in a Jeol 100 CX II electron microscope.

We used HPLC for corticosterone assays (Haughey & Jusko 1988, Swart *et al.* 1988) with absorbance detection at 254 nm. Aldosterone and plasma renin activity were determined by RIA (commercial kits purchased from Sorin Biomedica, Italy; intra-assay and interassay variations for aldosterone were 9·7% and 11·5% respectively and for plasma renin activity 7·6% and 9·1%). All experiments were carried out in duplicate. Mean values were compared by Student's *t*-test. A $P$ value of less than 0·05 was considered significant. In order to minimize circadian variations, all animals were killed and their trunk blood collected between 1400 and 1500 h.

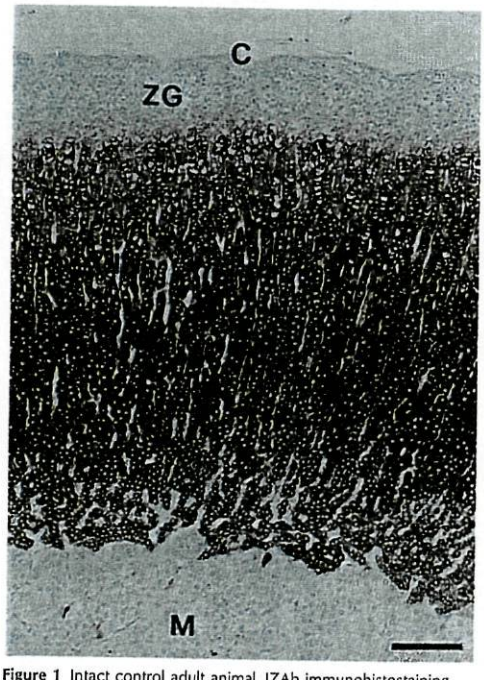


**Figure 1** Intact control adult animal. IZAb immunohistostaining (1:50); avidin–biotin complex technique. Zona glomerulosa is unstained. C, Capsule; ZG, zona glomerulosa; IN, inner zone (zona fasciculata+zona reticularis); M, medullary cells. Scale bar=100 μm.

## Results

As previously observed (Ho & Vinson 1993, Pignatelli *et al.* 1995), immunohistochemical staining with IZAb in intact control animals was restricted to the inner adrenocortical zones (zona fasciculata and reticularis) (Fig. 1). The zona glomerulosa and the medulla were unstained. No specific immunohistostaining was seen in the non-specific mouse IgG-treated control sections. Ultrastructurally, glandular elements showed the usual features of steroid-secreting cells, containing mitochondria with tubular and vesicular cristae, abundant profiles of smooth endoplasmic reticulum, and lipid droplets.

No significant morphological differences could be found in autotransplanted glands after chronic 0·9% saline solution or bFGF infusion when compared with autotransplanted controls. IZAb immunohistostaining was observed in almost all of the regenerated adrenocortical tissue (Fig. 2*a* and *b*), with the exception of some small clusters of cells beneath the capsular tissue and adjacent well-vascularized connective tissue septal layers. There was no clear zonation as seen in the intact animal, although the immunonegative cells, which appeared to be glomerulosa-like, occasionally formed a layer one to two cells thick. Ultrastructurally most cells showed fasciculata characteristics, with mitochondria containing typical vesicular cristae. Identical findings were also obtained in the subcapsular area, although here a few isolated cells with glomerulosa-type features could be seen.

After chronic administration of IGF-I, a remarkable difference in glandular architecture was seen. In these glands, a glomerulosa-like zone was clearly evident as an extended subcapsular layer some three to seven cells thick. This zone was devoid of immunostaining, in contrast with the IZAb- positive zona fasciculata-like cells that constituted the inner cell population (Fig. 3). Electron microscope findings revealed that cells in the inner zone continued to exhibit mitochondria with the vesicular cristae, lipid droplets and smooth endoplasmic reticulum profiles typical of fasciculata cells (Fig. 4). However, in the outer subcapsular area, this experimental group showed cells with mitochondria resembling those of immature or fetal glomerulosa cells. Although exhibiting smooth endoplasmic reticulum profiles with evidence of hypertrophy and a lipid droplet depletion, these glomerulosa-like cells were mainly characterized by the presence of mitochondria with irregular tubular or tubulovesicular cristae but

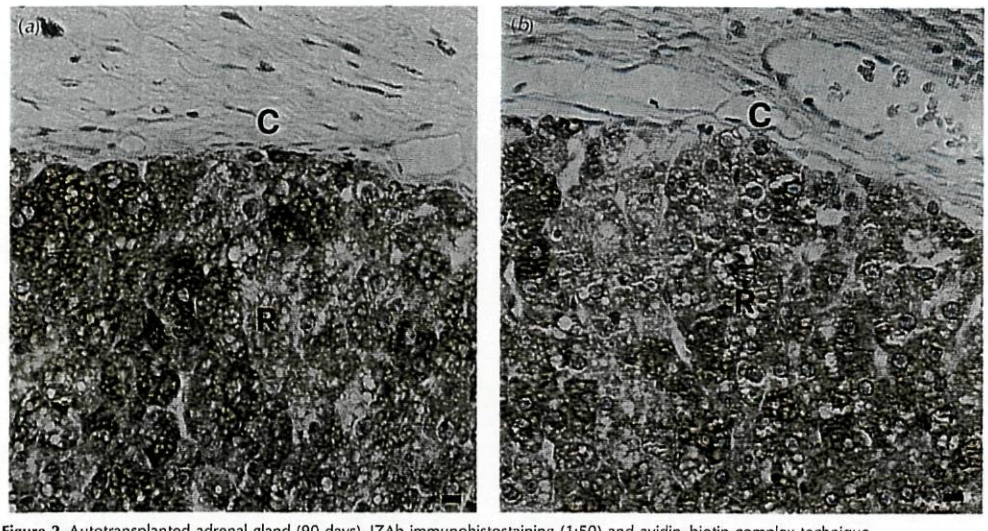


**Figure 2** Autotransplanted adrenal gland (90 days). IZAb immunohistostaining (1:50) and avidin–biotin complex technique. (*a*) Saline-treated animal. Staining is observed in almost all of the regenerated adrenocortical tissue. (*b*) bFGF-treated animal. Same immunohistostaining distribution as for (a) with no clear zonation as seen in Fig. 1. C, capsule; R, regenerated cortical tissue. Scale bar=10 μm.

lacking other features characteristic of reticularis cells, such as lipofuscin granules (Fig. 5). Chromaffin medullary tissue could not be found in any of the experimental procedures.

Prolonged infusion with IGF-I produced a significant rise in plasma aldosterone concentrations when compared with saline or bFGF infusion. Plasma renin activity was significantly decreased. In contrast, serum corticosterone was significantly lower in autotransplanted IGF-I-treated animals when compared with the other experimental groups (Table 1).

## Discussion

The growth and differentiation of the adrenal cortex continues to present challenges for our understanding of the mechanisms involved. Most authors now believe that the three major zones are not immutable, and that cellular transformation, for example from glomerulosa to the fasciculata, or fasciculata to reticularis, occurs under normal conditions, and indeed is a feature of the cellular life history as the cells migrate centripetally from the peripheral part of the gland. Cellular transformation, however, occurs only at the specific sites where the zones adjoin, and consequently the zones retain their relative positions within the gland despite changes in relative abundance that may reflect physiological stimulation. The difficulty lies not only in identifying the factors that account for such cellular transformation, but also in explaining why they act only at such specific locations.

As the parenchymal cells are thought to migrate through the cortex, it might be supposed that the tissue-organizing factors originate in other structures that retain their position. These might, for example, include elements of the vascular system (Rosolowsky & Campbell 1994, Rubin & Levin 1994), or the innervation (De Léan *et al.* 1984, Gallo-Payet *et al.* 1987, Rebuffat *et al.* 1988, Hinson *et al.* 1992, Vizi *et al.* 1992, 1993, Malendowicz 1993, Bornstein *et al.* 1994, Hinson *et al.* 1994), and considerable evidence now exists that both can greatly affect adrenocortical function under experimental conditions. However, when glands are autotransplanted, these elements are lost (even if only temporarily), and still under appropriate conditions adrenocortical zonation can occur. Therefore the factors that regulate adrenocortical zonation must have another source.

The adrenal autotransplantation model has proved to be an invaluable experimental tool, which clearly shows the presence of local autocrine/paracrine mechanisms in addition to systemic regulators (Wilkinson *et al.* 1981, Belloni *et al.* 1991, Zieleniewski & Zieleniewski 1995, Zieleniewski *et al.* 1995, Vendeira *et al.* 1996*a*, Malendowicz 1997).

After chronic stimulation with IGF-I, the adrenal neocortex exhibited an extended subcapsular area corresponding to the zona glomerulosa, and devoid of IZAb

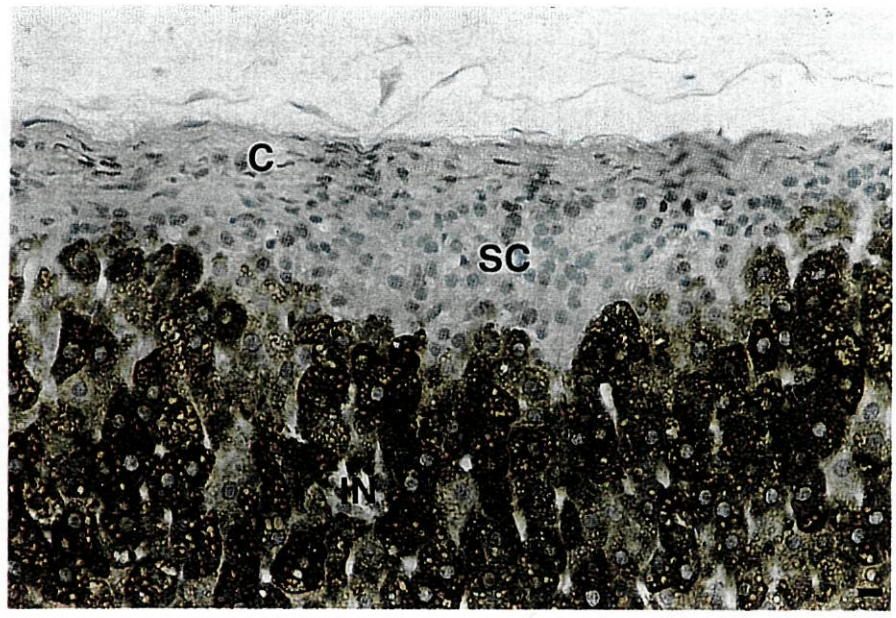


**Figure 3** Autotransplanted adrenal gland (90 days). IZAb immunohistostaining (1:50) and avidin–biotin complex technique. IGF-I-treated animal. A glomerulosa-like zone devoid of immunohistostaining is clearly evident. C, Capsule; SC, subcapsular glomerulosa-like zone; IN, inner zone. Scale bar=10 μm.

immunoreactivity. The suggestion that IGF-I stimulates the zona glomerulosa is supported by a significant rise in plasma aldosterone concentrations. It is known from other studies that IGF-I exerts a potent proliferative effect on adrenocortical cells *in vitro* (Naaman *et al.* 1989), and furthermore that IGF-I is also synthesized within adrenal tissue, which contains a relative abundance of both IGF-I mRNA and the peptide (Hansson *et al.* 1988, Ho & Vinson 1995). IGF-I also stimulates steroidogenesis in isolated adrenocortical cells (Penhoat *et al.* 1994, Weber *et al.* 1997) and has been implicated in compensatory growth after unilateral adrenalectomy, as well as in adrenal regeneration and differentiation after bilateral adrenal enucleation (Jackson *et al.* 1991). Using non-radioactive *in situ* hybridization, IGF-I mRNA was located mainly in the zona fasciculata and adrenal medulla in untreated animals (Ho & Vinson 1995). After ACTH stimulation or sodium restriction, the translation of IGF-I mRNA is enhanced in zona glomerulosa, also supporting a local proliferative role for IGF-I. This seems plausible as IGF-I receptors have been identified in rat, bovine and human adrenal glands (Penhoat *et al.* 1988, Shigematsu *et al.* 1989, Arafah 1991, Weber *et al.* 1995, 1997). In our experiments, IGF-I administration increased the extent of IZAg non-expressing cells in the subcapsular area, suggesting its particular modulatory role in the peripheral areas of the regenerated adrenal cortex. This is in a sense similar to previous findings of IGF-I mRNA localization in the fetal adrenal (Han *et al.* 1987, Mesiano *et al.* 1993) in which a predominantly capsular localization was observed. The low plasma corticosterone

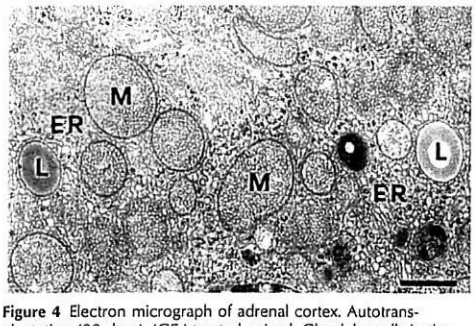


**Figure 4** Electron micrograph of adrenal cortex. Autotransplantation (90 days). IGF-I-treated animal. Glandular cells in the inner zone exhibited mitochondria (M) with vesicular cristae, lipid droplets (L) and smooth endoplasmic reticulum profiles (ER), typical of fasciculata cells. Scale bar=1 μm.

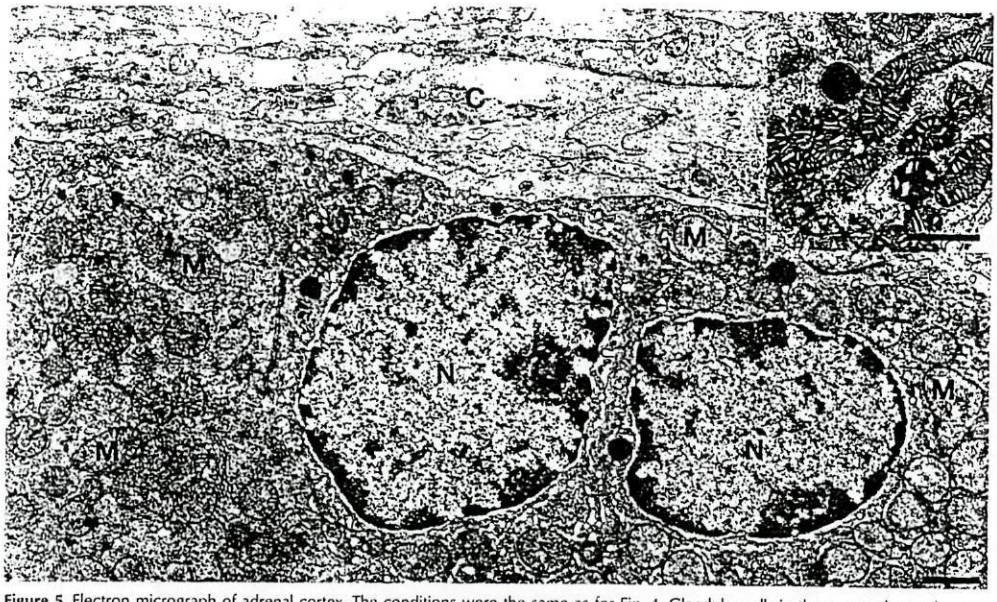


**Figure 5** Electron micrograph of adrenal cortex. The conditions were the same as for Fig. 4. Glandular cells in the outer subcapsular area exhibit mitochondria with irregular cristae resembling those of the fetal glomerulosa cells. Note the absence of lipid droplets. C, capsule; N, nucleus; M, mitochondria. Scale bar=1 μm. Inset: details of regular mitochondrial cristae from glomerulosa cells of intact rats. Scale bar=1 μm.

concentrations observed after chronic administration of IGF-I deserve a comment, because they do not appear to be consistent with the morphological evidence. This is, however, a common finding in adrenal-regenerated autotransplants, a situation in which a high plasma ACTH concentration is also observed (Wilkinson *et al.* 1981, Engeland 1984).

The morphological and biochemical analysis of adrenal grafts submitted to chronic bFGF administration showed some unexpected effects when compared with previous results. In fact, our observations revealed that bFGF produced no change in IZAg expression when compared with control autotransplanted animals. Immunohistostaining was distributed in almost all of the regenerated adrenocortical tissue, with the exception of some small clusters of subcapsular cells. Interestingly, Basile & Holzwarth (1993, 1994) showed that, in the unilaterally adrenalectomized animal, in the remaining gland bFGF was preferentially located in the outer cortical area and adrenal medulla, while the expression of bFGF receptors was predominantly in the capsule and zona glomerulosa. Hence, bFGF may play a role in compensatory adrenal regeneration. In this study, we observed that plasma aldosterone concentrations were significantly lower than in control autotransplanted animals, and this finding suggests that bFGF partly replicates the actions of chronic ACTH in normal animals. The invariable plasma renin activities, when compared with controls, are intriguing,

**Table 1** Endocrine effects of prolonged infusion (7 days) with saline solution (SS), bFGF or IGF-I in autotransplanted (AT) rats. The data presented are means ± S.E.M.

| | Intact rat | AT+SS | AT+bFGF | AT+IGF-I |
|---|---|---|---|---|
| Plasma aldosterone (pg/ml) | 290 ± 59·1$^{(a)}$ | 81·15 ± 14·3$^{(d)}$ | 18·5 ± 8·8$^{(g)}$ | 132·4 ± 10·8$^{(j)}$ |
| Serum corticosterone (μg/ml) | 0·19 ± 0·02$^{(b)}$ | 0·09 ± 0·003$^{(e)}$ | 0·1 ± 0·01$^{(h)}$ | 0·05 ± 0·004$^{(k)}$ |
| Plasma renin activity (ng/ml per h) | 22·79 ± 3·54$^{(c)}$ | 23·75 ± 3·81$^{(f)}$ | 18·37 ± 2·32$^{(i)}$ | 11·47 ± 3·46$^{(l)}$ |

Statistical comparison of the data: a vs *d*, $P$=0·001; *b* vs *e*, $P$=0·005; *c* vs *f*, n.s.; *d* vs *g*, $P$=0·01; *e* vs *h*, n.s.; *f* vs *i*, n.s.; *d* vs *j*, $P$=0·01; e vs *k*, $P$=0·01; *f* vs *l*, $P$=0·01.

suggesting that even after a 7 day infusion of bFGF, the renin–angiotensin–aldosterone system still lacks adequate regulatory feedback.

As bFGF is also a potent angiogenic and neurotrophic factor (Flamme & Risau 1992, Bikfalvi *et al.* 1997), it has been suggested that it may have a modulatory role in vascularization and innervation of the adrenal gland (Basile & Holzwarth 1994). We postulate that it may have a role in the neovascularization process in the autotransplanted adrenal cortex, which we have already described (Vendeira *et al.* 1992, 1996*a*), although as previously noted, neural pathways were not observed in the autotransplanted gland at least in the early steps of regeneration.

These findings strongly suggest that growth factors have an important role in regulating adrenocortical zonation. The factors that influence the extent and sites of their expression as well as those of their receptors will now require further study in order to firmly establish the mechanisms of proliferation and steroidogenic differentiation in the regenerating gland.

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# CONSIDERAÇÕES FINAIS

# 1. Autotransplante da glândula supra-renal

## *1.1. Comentários Gerais*

Os mecanismos que regem a regeneração e a diferenciação dos tecidos endócrinos em geral e da supra-renal em particular são múltiplos, complexos e ainda hoje pouco claros.
Na presente dissertação, procuramos descrever e clarificar as alterações morfológicas e funcionais observadas no decorrer da regeneração e diferenciação adrenocortical após autotransplante da glândula supra-renal, bem como elucidar os mecanismos moduladores envolvidos neste processo.

Este modelo experimental de autotransplante já efectuado desde há várias décadas (Wyman e Suden, 1932; Ingle e Higgins, 1938a; Brenner e col., 1953; Penney e col., 1963; Taki e Nickerson, 1985; Belloni e col., 1990; Sarría e col., 1995), foi inicialmente desenvolvido com o intuito de estudar morfologicamente o processo de regeneração adrenocortical após a privação da medula supra-renal. O rato como animal de experiência cedo se revelou ideal para o estudo, dada a alta taxa de sucesso do enxerto quando comparada com a taxa observada em animais superiores incluindo o homem (Wyman e Suden, 1932; Murakami e Takahashi, 1982; Saxe e Connors, 1985; Okamoto e col., 1996). O local de eleição para a colocação do enxerto é extremamente variável, como já previamente referido. A utilização por nós efectuada do plano subcutâneo da região dorsal foi fruto da necessidade de criar um plano de fácil acesso e de execução técnica simples e rápida, e que simultaneamente permitisse ultrapassar as dificuldades inerentes a uma regeneração deficitária, por exemplo, por ineficácia do suporte vascular ou por condições locais pouco satisfatórias.

O córtex supra-renal adulto possui a capacidade de, em determinadas condições adversas (autotransplante ou enucleação), sofrer um processo proliferativo bem como de diferenciação esteroidogénica (Wyman e Suden, 1932; Ingle e Higgins, 1938a,b; Greep e Deane, 1949; Taki e Nickerson, 1985). Curiosamente, a utilização de populações constituídas por células do estroma, parenquimatosas e endoteliais adrenocorticais em cultura revelou o mesmo tipo de potencialidades (Roskelley and Auersperg, 1993), na ausência de elementos nervosos e/ou cromafins, permitindo reforçar a constatação da independência do enxerto em relação a estes factores.

Contudo, no animal intacto, o córtex supra-renal recebe inervação autónoma que afecta o ritmo de proliferação cortical e estimula a esteroidogénese (Dallman e col., 1976, 1980; Holzwarth e col., 1987; Holzwarth, 1988; Ehrhart-Bornstein e col., 1998). Para além de adrenalina e noradrenalina, a medula supra-renal contém e liberta um número variado de peptídeos reguladores passíveis de exercerem um efeito estimulador ou inibidor sobre as células adrenocorticais (Saria e col., 1980; Kondo e col., 1980; Kondo, 1985; Majane e col., 1985; Nussdorfer e col., 1988; Maubert e col., 1990, 1993; Nussdorfer, 1996), o que permite questionar sobre as consequências da sua ausência no modelo de autotransplante. Dentro destes neuropeptídeos, a substância P, a neurotensina, o peptídeo intestinal vasoactivo e o neuropeptídeo Y parecem exercer um importante papel no aumento da secreção de aldosterona (Mazzocchi e Nussdorfer, 1987; Nussdorfer e col., 1988; Cunningham e Holzwarth, 1988; Rebuffat e col., 1988, 1994; Malendowicz e col., 1992; Mazzocchi e col., 1996a; Renshaw e col., 2000), e a estimulação crónica com estes três últimos peptídeos induz o crescimento da zona glomerulosa (Mazzocchi e col., 1987, 1993a; Rebuffat e col., 1988); a oxitocina estimula apenas a actividade mitótica na zona glomerulosa (Payet e Isler, 1976; Stachowiak e col., 1995). Hinson e col. (1990) sugerem que o tecido adrenocortical constitui inicialmente um alvo para neurónios adrenomedulares pós-ganglionares, mas, à medida que córtex e medula se tornam progressivamente associados e a função da medula como gânglio se modifica, as fibras pós-ganglionares terminam inteiramente no interior da glândula, ora formando o tecido cromafim, ora inervando as células adrenocorticais.

De acordo com Dallman e col. (1976) e Gragg e Soliman (1993), as conexões nervosas parecem estar implicadas no crescimento adrenocortical compensatório e este facto constitui mais um importante elemento na avaliação da presença destes diversos neuropeptídeos nos nervos do córtex supra-renal (Holzwarth, 1984; Holzwarth e col., 1987; Malendowicz, 1993; Hinson e col., 1994a,b; Vinson e col., 1994; Toth e Hinson, 1995; Nussdorfer, 1996), com especial relevo no córtex externo, suportando a hipótese de uma regulação parácrina.

Estes achados clarificam e apoiam o papel da inervação cortical no controlo da proliferação, estrutura e função do córtex supra-renal, quer no animal intacto quer na resposta adrenocortical compensatória. Em apoio a esta teoria, Bornstein e col. (1994), utilizando o animal intacto, e através de um procedimento de localização imunohistoquímica para a proteína neuroendócrina cromogranina-A, identificou a presença de células cromafins em todas as zonas corticais incluindo a glomerulosa.

No entanto, mesmo na ausência de tecido nervoso ou cromafim, os processos regenerativos desenvolvem-se permitindo a sobrevivência e desenvolvimento do enxerto adrenocortical, tal como demonstrámos no primeiro trabalho, observando-se ainda critérios objectivos bioquímicos que suportam esta funcionalidade a nível dos mecanismos da esteroidogénese. Curiosamente, e apenas no primeiro trabalho, observámos um animal que, após 7 dias de autotransplante, mostrou a presença de células com características ultrastruturais de células cromafins. O significado deste facto permanece desconhecido, não se tendo observado alterações morfológicas ou funcionais a nível cortical. Este achado foi também previamente descrito por alguns autores (Wyman, 1928; Turner, 1939; Coupland, 1957, 1958), em contaste com a grande maioria dos trabalhos nesta área. Também em nenhum outro protocolo experimental por nós utilizado, com o autotransplante da glândula, voltamos a verificar tal facto, quer nos estudos em microscopia óptica clássica quer nos estudos ultrastruturais. Recentemente, Ulrich-Lai e Engeland (2000), em estudos envolvendo o autotransplante de tecido adrenocortical na região subcapsular renal, investigaram os fenómenos de reinervação do enxerto, tendo verificado que nesta localização é possível observar a existência

de fibras nervosas *de novo*, concluindo, de uma forma global, que os autotransplantes de glândula supra-renal não constituem um modelo válido de tecido adrenocortical desnervado. A escolha específica deste local de autotransplante pelos referidos autores poderá justificar a presença de factores locais facilitadores desta reinervação; no entanto, deve ter-se em conta que o tecido adrenocortical, dada a sua grande concentração de hormonas glicocorticóides, constitui um excelente meio para a sobrevivência de células nervosas transplantadas (Suhonen e col., 1990), actuando assim como um factor facilitador da reinervação.

No modelo por nós utilizado e no autotransplante em geral, bem como na enucleação, a ausência de regeneração de tecido medular é a regra, tal como previamente observado e posteriormente confirmado por outros autores (Ingle e Higgins, 1938a,b; Skelton, 1959; Belloni e col., 1990,1991; Sarría e col., 1995; Nabishah e col. 1998). Assim, e tal como já referido, a influência cortical dos nervos autónomos e das células cromafins é inexistente, o que implica que o enxerto adrenocortical cresce e diferencia-se, segregando corticosteróides apenas sobre a influência da ACTH e de outras prováveis hormonas tróficas (Gibson e Krieger, 1981).

Como é sabido, as concentrações plasmáticas de ACTH estão cronicamente aumentadas após supra-renalectomia bilateral e autotransplante (Gibson e Krieger, 1981; Wilkinson e col., 1981; Engeland, 1984; Belloni e col., 1990, 1991), a que não é alheio o facto de os níveis plasmáticos de corticosterona observados constituirem cerca de 50% dos registados no animal intacto. Estivariz e col. (1982, 1988a) sugerem a possibilidade da participação de outros factores com relevo na mitogénese cortical e salientam o papel de peptídeos derivados da pro-opiomelanocortina (POMC), capazes de reverter parcialmente a atrofia glandular observada em animais hipofisectomizados e submetidos a enucleação supra-renal bilateral, situação não observada pela administração de ACTH (Estivariz e col., 1988b). Curiosamente, a detecção destes peptídeos foi efectuada na medula (Thorne e col., 1991), fornecendo assim novo suporte para a regulação parácrina entre os dois

tecidos supra-renais (Szalay, 1993), e a existência de mecanismos de "feed-back" entre os corticosteróides sintetizados de novo e a glândula hipofisária, após enucleação, permitiu reforçar o papel destes peptídeos nos processos da regeneração precoce (Perone e col., 1997).

Independentemente da contribuição das estruturas nervosas e dos peptídeos de origem hipofisária nos processos de regeneração e diferenciação adrenocortical, é deveras pertinente salientar que, no modelo de autotransplante, e na ausência ou na presença de fibras nervosas como recentemente descrito, observa-se uma recuperação incompleta das concentrações plasmáticas de aldosterona e corticosterona na presença de concentrações suprafisiológicas de ACTH como já referido. Da mesma forma, é também importante frisar que a zonação clássica adrenocortical, tal como a conhecemos no animal intacto, não é observada no autotransplante, apesar dos primeiros estudos por nós efectuados (trabalho 1) e de outros similares (Penney e col., 1963), nos terem induzido a considerar idêntica à do animal intacto. De facto, os estudos em microscopia óptica com colorações de rotina (H+E) e os estudos ultrastruturais não revelaram alterações significativas ao fim de 90 dias de autotransplante, nomeadamente nas observações feitas nas células glandulares de características fasciculadas, que, como sabemos, constituem a maior componente celular do enxerto.

### *1.2. Regeneração e Diferenciação no Autotransplante*

Os resultados do presente trabalho suscitam algumas questões e duas das mais pertinentes dizem respeito, por um lado, à localização da resposta proliferativa no autotransplante e por outro, ao desenvolvimento da zonação adrenocortical quer morfológica quer funcional. Os estudos autorradiográficos exibem marcação pela timidina tritiada em localização subcapsular no que diz respeito à população de células glandulares. Observações prévias (Skelton, 1959; Seki e col., 1969) sugerem que o processo proliferativo tem origem exclusiva na periferia do enxerto. No

entanto, deve salientar-se que nas fases precoces do enxerto existe uma abundante marcação autorradiográfica de células de tipo fibroblástico, provavelmente resultante de uma reorganização a nível do tecido conjuntivo (trabalho 1), sem no entanto se observar qualquer tipo de modificação neste tipo de células que permita sugerir uma participação da cápsula ou tecido conjuntivo adjacente nos processos de regeneração. Os nossos dados sugerem que o tecido glandular subcapsular é o responsável pela actividade de regeneração, observando-se a maior marcação nuclear, por timidina tritiada, entre o 7º e o 15º dia pós-autotransplante.

O procedimento de regeneração é acompanhado de fenómenos de angiogénese dada a exuberante neovascularização observada na periferia do enxerto, o que levanta questões não só no que diz respeito à angiogénese propriamente dita, mas também ao papel do endotélio nos fenómenos proliferativos. A este respeito é deveras curioso o facto do arranjo da vascularização adrenocortical no rato intacto ser tal que permite que cada célula glandular esteja em contacto directo com vasos sanguíneos (Pudney e col., 1981; Vinson e col., 1985; Vinson e Hinson, 1992; Tokunaga, 1996; Basset e West, 1997), o mesmo se passando após os fenómenos de angiogénese no autotransplante. Apesar de os mecanismos de angiogénese continuarem pouco claros, devemos, no entanto, ter em conta a expressão aumentada de FGF-2 na glândula contralateral após adrenalectomia e nomeadamente nas regiões externas assim como uma expressão aumentada de receptores para FGF-2 na cápsula e zona glomerulosa (Basile e Holzwarth, 1993, 1994). Este facto, aliado ao conhecimento de que o FGF-2 é um potente factor angiogénico (Folkman e Shing, 1992; Flamme e Risau, 1992; Folkman e D'Amore, 1996; Bikfalvi e col., 1997), sugerem um papel relevante deste peptídeo na resposta angiogénica necessária à neovascularização observada nas fases iniciais do autotransplante de glândula supra-renal, e, portanto, à facilitação do acesso a nutrientes e outros factores de crescimento com consequências relevantes no desencadear dos mecanismos de regeneração e diferenciação. Da mesma forma, a expressão do FGF-2 bem como do factor de crescimento endotelial vascular (VEGF) parece ser regulada pela acção da ACTH bem

como pela acção de factores locais solúveis (Risau, 1995, 1998; Shifren e col., 1998; Gaillard e col., 2000), podendo constituir um factor importante quer na manutenção do endotélio adrenocortical adulto, quer na neovascularização observada após estímulo regeneratório desencadeado por transplante (Thomas e col., 1997; Thomas e Hornsby, 1999).

A este respeito, a integração entre os sistemas imunitário e neuroendócrino deve ser mencionada, dada a existência de argumentos favorecendo o papel das citoquinas na estimulação dos mecanismos da angiogénese (Imura e col., 1991; Sachs, 1992; Reichlin, 1993; Ehrhart-Bornstein e col., 1996; Bornstein e Vaudry, 1998; Marx e col., 1998). Numerosos estudos envolvendo a acção da interleucina-1 na glândula supra-renal sugerem a existência de um mecanismo estimulador, permitindo um aumento da secreção de corticosterona (Andreis e col., 1991a; Gwosdow e col., 1992; Rebuffat e col., 1992; Mazzocchi e col., 1993b). De acordo com Zieleniewski e col. (1995), a interleucina-1 pode exercer um papel estimulador nos fenómenos mitóticos do tecido adrenocortical regenerado após enucleação, contribuindo, juntamente com os fenómenos de angiogénese, para uma facilitação do crescimento glandular na fase inicial do período pós-autotransplante.

O estudo dos fenómenos de neovascularização, na fase inicial, revelou simultaneamente a presença de múltiplos processos de necrose observados nas áreas internas corticais, seguindo-se um crescimento celular centrípeto com provável origem nas células glomerulosas subcapsulares, de acordo com os dados referidos. Esta hipótese de que as células parenquimatosas migram através do córtex durante os processos de proliferação e posterior diferenciação (teoria da migração celular), leva a prever, no entanto, a existência de factores organizadores tecidulares que fixem as suas posições na arquitectura cortical, permitindo exercer a sua actividade parácrina em localizações específicas com influência no controle mitótico e da esteroidogénese quer no animal intacto quer após estímulo proliferativo como no modelo de autotransplante (Ottenweller e Meier, 1982; Hinson e col., 1985, 1986a; Vinson e Ho, 1998a; Vinson e col., 1998). Alguns destes factores já

foram previamente abordados nomeadamente a inervação cortical, a presença de tecido cromafim em território cortical e a presença de neuropeptídeos com actuação parácrina cortical expressos em células cromafins e/ou nervosas (De Léan e col., 1984; Gallo-Payet e col., 1987; Rebuffat e col., 1988; Hinson, 1990; Hinson e col., 1992; Vizi e col., 1992, 1993; Malendowicz, 1993; Bornstein e col., 1990, 1991, 1992, 1994, 1997; Hinson e col., 1994a,b, 1996a, 1999; Toth e Hinson, 1995; Vinson e col., 1994; Zieleniewski e Zieleniewski, 1995; Guillon e col., 1995; Hinson e Kapas, 1996; Toth e col., 1997; Janossy e col., 1998; Szalay e col., 1998; Haidan e col., 1998; Hochol e col., 1999).

Outros factores com provável influência na proliferação e diferenciação adrenocortical incluem os peptídeos de origem hipofisária, também já mencionados. A este respeito, e para além da ACTH e dos peptídeos derivados da pro-opiomelanocortina (POMC), existem algumas referências, ainda pouco clarificadas, sobre o papel da hormona libertadora de corticotrofina (CRH) no controle da esteroidogénese supra-renal. De facto, desde a identificação deste peptídeo na glândula supra-renal e sua secreção em resposta à estimulação do nervo esplâncnico (Hashimoto e col., 1984; Bruhn e col., 1987; Minamino e col., 1988; Edwards e Jones, 1988; Oers e col., 1992), vários trabalhos têm referido uma potencial importância desta hormona na indução da libertação de ACTH pelas células medulares (Jones e Edwards, 1990; Andreis e col., 1992; Mazzocchi e col., 1997a), introduzindo o conceito de um sistema CRH-ACTH intramedular provavelmente sujeito a regulação nervosa (Andreis e col., 1991b; Jones e Edwards, 1992; Markowska e col., 1993), e condicionando um mecanismo de regulação parácrina cortical (Gallo-Payet e col., 1987; Hinson, 1990; Bornstein e col., 1990, 1991), com influência nos mecanismos da esteroidogénese, nomeadamente na via glicocorticóide. De acordo com estes factos, a ausência de tecido medular nos enxertos adrenocorticais provavelmente condicionará uma diminuição da actividade esteroidogénica tal como previamente sugerido por Belloni e col. (1990) e Andreis e col. (1992), justificando parcialmente as baixas concentrações de corticosterona verificadas neste modelo.

Ainda dentro destes factores, com provável importância reguladora parácrina adrenocortical, devem ser incluídos os elementos do sistema vascular, nomeadamente a endotelina-1 (Hinson e

col., 1986b; Vane e col., 1990; Rosolowsky e Campbell, 1990, 1994; Rubin e Levin, 1994; Hinson e Kapas, 1998; Rosolowsky e col., 1999), o sistema renina-angiotensina local (Mulrow, 1989, 1992; Kon e col., 1990; Gupta e col., 1992; Wang e col., 1992; Sander e col., 1994; Rocco e col., 1994; Vinson e col., 1995, 1996; McEwan e col., 1996; Vinson e Ho, 1998b; Vinson e col., 1998), e o óxido nítrico (Afework e col., 1992; Breslow e col., 1993; Cameron e Hinson, 1993; Hinson e col., 1996b). Em relação ao papel deste último, a presença da enzima síntase do óxido nítrico já foi demonstrada na glândula supra-renal quer a nível cortical, quer a nível medular, e o seu papel como agente vasodilatador local sugere uma potencial acção a nível da esteroidogénese, à semelhança dos efeitos vasculares observados após administração de ACTH (Maier e Staehlin, 1968; Tait e col., 1987).

Da mesma forma, o sistema renina-angiotensina local está hoje reconhecido como mediador na resposta da zona glomerulosa a fenómenos de estimulação crónica, e o seu papel na regulação autócrina da síntese de mineralocorticóides deve ser considerado, dado o evidente aumento de CYP11B2 na zona glomerulosa após estimulação deste sistema em animais transgénicos exibindo o gene codificador da renina predominantemente na glândula supra-renal (Sander e col., 1994). No entanto, tal como em relação ao óxido nítrico, o seu papel nos fenómenos de regeneração e diferenciação pós-autotransplante permanecem por estudar.

Os factores de crescimento, nomeadamente o FGF-2 e o IGF-I merecem um relevo especial dada a possibilidade de se comportarem como amplificadores parácrinos, condicionando a actuação destes factores morfogénicos (Vinson e Ho, 1998a).

## 2. Endotelina-1 e factores de crescimento (FGF-2 e IGF-I)

Neste tipo de estudos, a importância do reconhecimento e localização perfeita das zonas do córtex supra-renal regenerado foi atentamente considerada, dado que a zonação cortical morfológica evidenciada apresenta, na maior parte das vezes, limites pouco nítidos, e, de facto, a migração celular observada em modelos de proliferação glandular adrenocortical acompanha-se de alterações morfológicas (Zajicek e col., 1986), que não reflectem necessariamente a sua especificidade funcional (Tangalakis e col., 1989; Ganguly, 1991).

Assim, estudámos as supra-renais intactas e autotransplantadas por microscopia óptica, em cortes sujeitos a coloração simples pela hemateína/eosina e em cortes submetidos a técnica de imunohistoquímica amplificada pelo método ABC. Esta foi efectuada pela necessidade de adoptar um método com maior capacidade discriminatória na análise de alterações celulares discretas, importantes nos estudos experimentais de regeneração adrenocortical. Utilizámos um anticorpo monoclonal - IZAb - que reage com um antigénio detectado somente na zona supra-renal interna (zona fasciculada + zona reticular), evidenciando uma zona desprovida de imunomarcação - zona glomerulosa (Laird e col., 1988; Barker e col., 1992; Ho e col., 1994).

A administração aguda de endotelina-1 utilizando a veia jugular (Renaud, 1969), em animais autotransplantados, revelou uma zona de tipo glomerulosa, morfologicamente bem evidente, de acordo com a marcação por IZAb, mas sem expressão significativa em estudos de microscopia de luz clássica (trabalho 3). Este facto não se observou nos grupos controlo, nem nos grupos autotransplantados mas desprovidos de estimulação. Paralelamente, observou-se um nítido e significativo aumento das concentrações plasmáticas de aldosterona e corticosterona (trabalhos

2 e 3). Malendowicz e col. (1997), utilizando como modelo de regeneração a enucleação glandular, confirmaram o efeito estimulador da endotelina-1 na actividade esteroidogénica adrenocortical, e referiram ainda um aumento do índice mitótico após administração deste peptídeo, sugerindo um efeito combinado na proliferação celular e na capacidade secretora, funções estas inevitavelmente presentes na história natural do tecido adrenocortical submetido a estímulo regenerador (Nussdorfer, 1986). Estes resultados, obtidos após a administração de um peptídeo segregado por células endoteliais (Yanagisawa e col., 1988), e com receptores bem caracterizados na zona glomerulosa supra-renal do rato e do homem (Davenport e col., 1989; Koseki e col., 1989; Kohzuki e col., 1989, 1991; Gomez-Sanchez e col., 1990; Belloni e col., 1994, 1996, 1997; Kapas e col., 1996; Rossi e col., 1997; Mazzocchi e col., 1996b, 1997b; Pecci e col., 1998; Rebuffat e col., 1999, 2000), apoiam a hipótese de que os factores locais constituem agentes preponderantes na indução e manutenção de uma zona glomerulosa funcional. De acordo com Masaki (1993) e Nussdorfer e col. (1999), a endotelina-1 é um factor importante na regulação local da produção de catecolaminas e esteróides na glândula supra-renal, e a esta hipótese não é alheio o facto de este peptídeo estimular a secreção de aldosterona (Cozza e col., 1989; Morishita e col., 1989; Delarue e col., 1990; Rosolowsky e Campbell, 1990; Woodcock e col., 1990a,b; Mazzocchi e col., 1990a; Hinson e col., 1991a,b,c; Takuwa, 1993; Nussdorfer e col., 1997), promover o crescimento volumétrico da zona glomerulosa e respectivas células glandulares (Mazzocchi e col., 1990b), e ainda de exercer um forte efeito proliferativo *in vivo* da zona glomerulosa por um mecanismo provavelmente similar à angiotensina II (Mazzocchi e col., 1992, 1997b; Nussdorfer e col., 1997). Os seus efeitos estão provavelmente envolvidos na regulação funcional desta zona, não esquecendo todavia que a estimulação com ACTH desencadeia um processo de vasodilatação adrenocortical (Maier e Staehlin, 1968) e promove um aumento da secreção de corticosterona (Nussdorfer e col., 1978; Nussdorfer, 1986; Tait e col., 1987), verificando-se simultaneamente um aumento de endotelina imuno-reactiva na veia supra-renal, o que permite sugerir um papel modulador deste peptídeo na resposta adrenocortical à estimulação com ACTH (Hinson e col., 1991a,c; Mazzocchi e col., 1998).

Esta hipótese constitui suporte para os nossos achados no que diz respeito ao aumento das concentrações plasmáticas de corticosterona após estimulação com endotelina-1.
No entanto, a possibilidade da estimulação de células adrenocorticais transicionais no que diz respeito ao fenótipo glomerulosa/fasciculada, pela administração de endotelina-1, deve também ser considerada. De facto, este tipo celular é descrito no animal intacto, ocupando habitualmente o lugar da denominada zona de transição (Cater e Lever, 1954; Gomez-Sanchez e col., 1988). Segundo Mitani e col. (1994, 1999), trata-se de uma zona inerte do ponto de vista esteroidogénico dada a ausência de CYP11B2 e CYP11B1. No entanto, a sua condição posicional a nível da arquitectura adrenocortical implica provavelmente um maior dinamismo zonal, e portanto uma maior sensibilidade a factores reguladores locais, incluindo a endotelina-1.

A administração crónica de factores de crescimento (IGF-I e FGF-2), efectuada por via peritoneal e dependente da actividade de minibombas osmóticas (trabalho 4), mostrou dados relevantes no estudo da diferenciação adrenocortical pós-autotransplante. De facto, a utilização de FGF-2 parece reproduzir as acções da ACTH no animal intacto dada a deficiente capacidade funcional observada a nível da zona glomerulosa, expressa, por um lado, pela excessiva imunomarcação pelo IZAb e, por outro lado, pela diminuição muito significativa das concentrações plasmáticas de aldosterona, sem alteração da corticosterona. Contudo, a administração de IGF-I revelou uma expressão acentuada do fenótipo secretor tipo glomerulosa e uma diminuição do tipo fasciculada/reticular, possivelmente simulando a acção observada após administração de angiotensina-II ou a restrição de sódio na dieta. Estes resultados foram traduzidos pelo aumento significativo das concentrações plasmáticas de aldosterona e pela diminuição da corticosterona, bem como pela exibição de uma zona subcapsular tipo glomerulosa desprovida de imunomarcação pelo IZAb.
Como já foi sugerido, o papel destes dois factores pode ser analisado tendo em vista um efeito regulador a nível parácrino, amplificando a acção de outros sistemas envolvidos na proliferação e/ou diferenciação adrenocortical (L'Allemand e col., 1996; Ho e Vinson, 1997; Vinson e Ho, 1998a;

Le Roy e col., 2000). De facto, e de acordo com Ho e Vinson (1995), a administração de ACTH e a restrição de sódio activam a expressão genética de FGF-2 e IGF-I na zona glomerulosa, fornecendo suporte a esta hipótese. Em 1991, Mesiano e col. estudando a glândula supra-renal fetal, já haviam admitido que a acção do FGF-2 seria mediada pela ACTH, e, do mesmo modo, Penhoat e col. (1989) e Pham-Huu-Trung e col. (1991) sugeriram um papel do IGF-I na estimulação da diferenciação adrenocortical após administração de ACTH ou angiotensina-II. Esta acção local é apoiada por diversos estudos demonstrando a presença de receptores específicos para estes factores no tecido adrenocortical (Penhoat e col., 1988; Shigematsu e col., 1989; Arafah, 1991; Jaye e col., 1992; Basile e Holzwarth, 1993, 1994; Weber e col., 1995, 1997).

A importância da investigação destes dois factores decorre do facto de numerosos dados experimentais suportarem uma acção importante no crescimento e regulação de diversos sistemas biológicos (Goustin e col., 1986; Han e col., 1987; Grothe e Unsicker, 1989; Roith, 1997; Bikfalvi e col., 1997; Ray e Melmed, 1997), e daí, a sua função mitogénica e de regulação da esteroidogénese a nível do córtex supra-renal dever ser considerada, tal como foi já previamente referido (Gospodarowicz e col., 1977; Naaman e col., 1989; Bergh e col., 1991; Mesiano e col., 1993; Viard e col., 1993; Penhoat e col., 1994; Weber e col., 1997).

Neste contexto, o estudo das alterações induzidas pela administração destes factores, em modelos de regeneração e diferenciação adrenocortical, necessita de uma investigação mais aprofundada. Os resultados por nós observados, juntamente com os dados de Jackson e col. (1991), que referiram a secreção de IGF-I por células adrenocorticais em situação de proliferação induzida por supra-renalectomia unilateral ou enucleação, sugerem um papel relevante do IGF-I nestes modelos de regeneração adrenocortical, tal como já observado em modelos de regeneração renal compensatória, angiogénese e regeneração muscular (Stiles e col., 1985; Lajara e col., 1989; Hansson e col., 1989; Sommerland e col., 1989).

## 3. Perspectivas

De acordo com Viard e col. (1993, 1994) e Vinson e Ho (1998a), a importância dos factores de crescimento não se limita, no entanto, à amplificação de sinais morfogénicos, mas também à regulação de factores de transcrição, conduzindo desta forma à expansão de proto-oncogenes localizados a nível adrenocortical com influência na sua capacidade proliferativa e esteroidogénica. De facto, a administração de ACTH leva a um aumento da expressão das fosfoproteínas codificadas pelos proto-oncogenes c-fos e c-jun no tecido adrenocortical (Yang e col., 1989, 1990; Koistinaho e col., 1990; Ohno e col., 1992; Lehoux e Ducharme, 1995). Estes achados revestem-se de particular interesse dado que no modelo de autotransplante existe sistematicamente um aumento das concentrações plasmáticas de ACTH, cujas repercussões fisiológicas à luz dos sistemas reguladores mencionados devem ser criteriosamente estudadas. Assim, a determinação imunohistoquímica das proteínas expressas por estes proto-oncogenes devem ser pesquisadas nos modelos de regeneração, quer em condições basais, comparando com o animal intacto, quer em situações de estimulação com factores de crescimento, com vista à obtenção de um eventual padrão de expressão dos proto-oncogenes, relacionado com diferentes estados morfofuncionais observados nas sucessivas etapas da proliferação e diferenciação adrenocortical.

Na mesma linha de investigação, o estudo da expressão da CYP11B2 e da CYP11B1 por métodos imunohistoquímicos, bem como a hibridização *in situ* de algumas destas enzimas CYP, têm vindo a revelar-se como dois excelentes métodos de avaliação do desenvolvimento do tecido adrenocortical, bem como da localização preferencial destas actividades enzimáticas, permitin-

do simultaneamente um estudo funcional glandular e uma capacidade discriminatória mais precisa da zonação morfofuncional (Chou e col., 1991; Sasano, 1992; Mitani e col., 1997). LeHoux e col. (1995), para além da localização de CYP11B2 na zona glomerulosa, demonstraram a expressão desta enzima nas membranas mitocondriais por imunohistoquímica ultrastrutural utilizando o ouro coloidal, o que permitiu um estudo enzimático preciso e uma vigilância da expressão de CYP11B2 após estimulação com angiotensina II ou restrição de sódio na dieta. Esta última situação permitiu a estes autores observarem um significativo aumento da imunomarcação para esta enzima nesta situação experimental. Em 1991, Müller e col., em estudos utilizando *Western blotting*, verificaram que a estimulação pela ACTH induzia uma diminuição da expressão de CYP11B2, compatível com os processos de "fascicularização" celular já previamente referidos e que são sobreponíveis com os estudos bioquímicos, morfológicos, imunohistoquímicos, e de hibridização *in situ* em microscopia de luz e electrónica, após a estimulação com este peptídeo (Gomez-Sanchez, 1985; Hornsby, 1987; Abayasekara e col., 1989; Ho e Vinson, 1993; Mitani e col., 1996). Belloni e col. (1989) efectuaram uma descrição cuidadosa sobre as alterações verificadas nas glândulas adrenocorticais acessórias após supra-renalectomia bilateral e confirmaram estes achados, verificando concentrações de aldosterona plasmática muito diminuídas paralelamente com concentrações de corticosterona progressivamente crescentes, e uma "fascicularização" celular ultrastrutural. É de salientar, no entanto, que para além das concentrações elevadas de ACTH que se evidenciam neste procedimento, é necessário ter em conta que as glândulas supra-renais acessórias são desprovidas de tecido medular, o que permite afirmar mais uma vez que o tecido medular supra-renal exerce um controle parácrino sobre a função da zona glomerulosa, tal como previamente discutido.

Estas observações permitem colocar sérias reservas quanto ao valor da zonação cortical estudada em microscopia óptica clássica.

A presença de um processo de "fasciculariza̧ção" celular deve também ser contemplada no modelo de autotransplante, dado que os nossos estudos imunohistoquímicos e de microscopia electrónica sobre a diferenciação do enxerto, permitem demonstrar que no tecido adrenocortical regenerado o tipo celular mais abundante apresenta características ultrastruturais de actividade esteroidogénica, nomeadamente a abundância de retículo endoplasmático liso, gotículas lipídicas e mitocôndrias com cristas vesiculares típicas da zona fasciculada, observando-se imunomarcação pelo IZAb na quase totalidade do enxerto (trabalhos 2, 3 e 4). Mitani e col. (1995), utilizando o modelo de enucleação bilateral, demonstraram a presença imunohistoquímica das enzimas CYP11B2 e CYP11B1 no tecido adrenocortical regenerado com expressão global semelhante à obtida por nós com o IZAb, traduzindo uma massa de tecido adrenocortical regenerado constituído fundamentalmente por células glandulares com características de tipo fasciculada e expressando fundamentalmente CYP11B1. Teebken e Scheumann (2000) efectuaram o transplante de células glomerulosas previamente submetidas ou não a cultura, na região subcapsular renal do rato. Estes autores obtiveram resultados que sugerem a teoria da migração celular como a explicação mais adequada aos mecanismos de zonação adrenocortical, e defendem que as células glomerulosas adquirem o fenótipo da zona fasciculada durante a migração centrípeta.

É importante salientar que os estudos clássicos de microscopia de luz, no nosso modelo, não permitem concluir, com segurança, sobre a diferenciação dos tipos celulares dadas as alterações zonais evidenciadas. Desta forma, a localização de CYP11B2 revela-se um objectivo importante neste modelo, dada a dificuldade observada quer a nível morfológico quer bioquímico, traduzida por concentrações deficitárias de aldosterona plasmática bem como a distorção zonal a nível da região subcapsular sem exibição de uma verdadeira zona glomerulosa desprovida de imunomarcação pelo IZAb (trabalhos 3 e 4).

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# SUMÁRIO E CONCLUSÕES

O autotransplante da glândula supra-renal do rato permite evidenciar os fenómenos de regeneração e diferenciação do tecido adrenocortical, na ausência de tecido medular. Este modelo envolve a colocação de fragmentos da glândula no plano entre a pele e músculo da região dorsal, com base num procedimento tecnicamente simples e de fácil reprodução, proporcionando um excelente método para o estudo da arquitectura e função adrenocorticais.
Utilizando estudos morfológicos, bioquímicos, autorradiográficos e imunohistoquímicos, obtivemos dados na tentativa de esclarecer alguns destes achados após o autotransplante.

Foi efectuada a análise dos enxertos adrenocorticais desde o início dos fenómenos proliferativos até à regeneração completa. Os estudos em microscopia de luz e electrónica permitiram observar as alterações evidenciadas nas células glandulares assim como no tecido conjuntivo envolvente.

Estudos autorradiográficos utilizando $^{3}$H-timidina mostraram uma expressão preponderante nas células glandulares subcapsulares. Foram ainda efectuados doseamentos hormonais plasmáticos de aldosterona e corticosterona, bem como a actividade da renina.

A endotelina-1, peptídeo de origem vascular, tem vindo a revelar-se como factor relevante nos processos de diferenciação celular, e o seu papel na estimulação da secreção de aldosterona no rato já foi demonstrado. O seu estudo como factor regulador local na estrutura e função da zona glomerulosa mereceu especial atenção no tecido adrenocortical autotransplantado. Uma vez que os estudos morfológicos utilizando a microscopia óptica convencional revelam dificuldades na correcta identificação dos tipos celulares adrenocorticais regenerados, utilizámos um método

com maior poder descriminativo para melhorar a análise das alterações celulares observadas. IZAb, um anticorpo monoclonal que reage com um antigénio (IZAg), localizado apenas na zona supra-renal interna, permitiu efectuar estudos imunohistoquímicos interessando à expressão zonal.

Da mesma forma, a fim de evidenciar um possível efeito de alguns factores de crescimento na diferenciação adrenocortical após o autotransplante de glândula supra-renal, efectuámos estudos imunohistoquímicos, ultrastruturais e bioquímicos após a administração do factor básico de crescimento fibroblástico (bFGF, FGF-2) e do factor de crescimento de tipo insulínico I (IGF-I), factores com actividade comprovada na proliferação celular e esteroidogénese adrenocortical.

Estes estudos possibilitam uma eventual contribuição para o esclarecimento da regulação local do tecido adrenocortical regenerado após autotransplante, incluindo os mecanismos da zonação morfológica bem como o potencial esteroidogénico da célula glandular adrenocortical.

Os resultados permitiram concluir que:

1. O autotransplante da glândula supra-renal do rato permite a obtenção de uma massa de tecido adrenocortical regenerado com viabilidade morfológica e funcional, e a sobrevivência do animal na ausência de corticoterapia de substituição.

2. Este procedimento no rato constitui um modelo de fácil execução, tecnicamente simples e reprodutível, possibilitando o estudo dos mecanismos de proliferação e diferenciação glandular adrenocortical, na ausência de tecido medular supra-renal.

3. A regeneração do tecido adrenocortical autotransplantado traduz-se em fenómenos proliferativos precoces com origem na periferia do enxerto, e de provável responsabilidade do escasso tecido glandular subcapsular.
4. Aos 90 dias após o autotransplante, o tecido adrenocortical regenerado exibe uma capacidade funcional inferior aos grupos controlo, não só na vertente glicocorticóide mas evidenciando, sobretudo, uma deficiente expressão bioquímica de hormonas mineralocorticóides.

5. Dada a importância da regulação parácrina evidenciada pela inervação e tecido medular na glândula supra-renal intacta, a aparente ausência de ambos no autotransplante assume-se como uma das principais causas das alterações morfofuncionais e zonais evidenciadas.

6. Os estudos imunohistoquímicos com IZAb permitem a distrinça de dois grupos celulares distintos no autotransplante tal como nos animais controlo, pelo que este anticorpo constitui um marcador para a identificação inequívoca dos tipos celulares adrenocorticais.

7. O IZAb reconhece um factor de localização específica (zona fasciculada + zona reticular), pelo que pode ser utilizado em estudos de zonação adrenocortical.

8. A administração aguda de endotelina-1 permite evidenciar uma estimulação da capacidade funcional da zona subcapsular de tipo glomerulosa, sugerindo uma acção moduladora local na regulação morfofisiológica desta zona.

9 - A administração de IGF-I revelou uma expressão acentuada do fenótipo secretor tipo glomerulosa. O seu reconhecido efeito amplificador sugere uma importante acção como mediador local secundário, com responsabilidade atribuída na diferenciação desta zona.

**10.** O FGF-2 reproduz as acções da ACTH no animal intacto, evidenciando um processo de "fascicularização" celular, tal como observado no autotransplante não estimulado. De acordo com as suas características funcionais, é de prever uma acção mais relevante nos processos de angiogénese que ocorrem durante a neovascularização nas fases precoces de regeneração do enxerto.

**11.** A zonação funcional nem sempre acompanha a zonação morfológica. De facto, é a diferenciação funcional entre os distintos tipos celulares adrenocorticais que possui importância biológica, nomeadamente a nível da esteroidogénese.

**12.** Os resultados apresentados suportam a ideia de que todas as células das diferentes zonas do córtex supra-renal pertencem ao mesmo tipo, exibindo características morfológicas e funcionais temporárias de acordo com a sua localização cortical, isto é, de acordo com o seu estado de diferenciação.

# SUMMARY AND CONCLUSIONS

Rat adrenal gland autotransplantation provides the regeneration and differentiation of adrenocortical tissue, in the absence of adrenal medullary cells. This model involves the placement of pieces of the adrenal gland in a dorsal plane between the skin and the muscle, and stands as a technically simple procedure, easy to reproduce method for the study of adrenocortical arquitecture and function.
Morphological, biochemical, autoradiographic and immunohistochemical data were obtained in order to understand these events after autotransplantation.

We examined the grafts since the early steps of proliferation until full regeneration was accomplished. Light and electron microscopic studies showed the changes in glandular cells as well as in the connective tissue, the labelling with $^{3}$H-thymidine showed a preponderant expression in subcapsular glandular cells, and plasma concentrations of corticosterone and aldosterone as well as renin activity were assayed.

A vascular product, endothelin-1 has been shown to have a role in cellular differentiation and to stimulate aldosterone secretion in rats. We examined the role of this peptide as a local regulatory factor in the structure and function of glomerulosa zone in autotransplanted adrenocortical tissue. In morphological studies with conventional light microscopy it is very difficult to clearly identify regenerated adrenocortical cell types, therefore a more discriminatory method was used for the analysis of the finest cellular alterations. IZAb, a specific antibody, which labels the antigen IZAg found only in the adrenal inner zone was used for immunohistochemical studies.

Also, to study the possible involvement of some growth factors, namely basic fibroblast growth factor (FGF-2) and insulin-like growth factor I (IGF-I), in adrenocortical cell differentiation, immunohistochemical, ultrastructural and biochemical studies were carried out on adrenal autotransplants.

These studies provide a possible contribution to the understanding of the local regulation of adrenocortical regenerated tissue, including the mechanisms of adrenocortical morphological zonation as well as the steroidogenic potencial of adrenocortical cells.

The following conclusions were drawn:

1. Rat adrenal gland autotransplantation provides the obtention of a regenerated adrenocortical mass that detains functional and morphological viability, leeding to the survival of the animal without daily steroid replacement.

2. In the rat, this procedure stands as a technically simple method, easy to reproduce model, that permits the study of the adrenocortical mechanisms of proliferation and differentiation, in the absence of adrenal medullary tissue.

3. Regeneration of autotransplanted adrenocortical tissue represents a sequence of precocious proliferative phenomena that take place at the graft's periphery, probably proceeding from subcapsular glandular cells.

4. 90 days after autotransplantation, regenerated adrenocortical tissue exhibits a functional capacity that is inferior to the control groups, not only at the glicocorticoid level, but also and more significant, a deficient biochemical expression of mineralocorticoid hormones.

5. According to the importance of paracrine regulation as depicted by the presence of nerves and medullary tissue in the intact adrenal, the absence of both structures in the graft stand as one of the most important reasons for the observed changes in adrenocortical morphophysiology and zonal arquitecture.

6. Immunohistochemical studies with IZAb showed the presence of two distinct cell groups in adrenal autotransplants, as well as in the control animals, therefore this antibody provides a specific marker for unequivocal identification of adrenal cell types.

7. IZAb recognizes a local-specific factor (fasciculata zone + reticularis zone), therefore it may be used to monitor adrenocortical zonation.

8. The acute administration of endothelin-1 leeds to the stimulation of the functional capacity of the subcapsular glomerulosa-like zone, suggesting a local modulator role in the morphophysiologic regulation of this zone.

9. The IGF-I administration showed an enhancement of the glomerulosa secreting phenotype. Its recognized amplifying effect suggests an important role as a secondary local mediator, with a preponderant action in zona glomerulosa differentiation.

10. FGF-2 administration replicates the actions of ACTH in the intact animal, exhibiting a process of cellular "fascicularization", as previously observed in the non-stimulated adrenal graft. According to its functional characteristics, a more relevant role is expected, concerning the angiogenic process thay occurs during the neovascularization processes, specially in the early start of adrenocortical regeneration.

**11.** Functional zonation does not always parallel morphological zonation. In fact, it is the functional differentiation between the distinct adrenocortical cellular types that retains the biological importance, specially concerning the steroidogenic processes.

**12.** These data support the hypothesis that all the cells from different adrenocortical zones share the same type, exhibiting temporary morphological and functional characteristics according to their cortical localization, that is to say, according to their differentiation status.