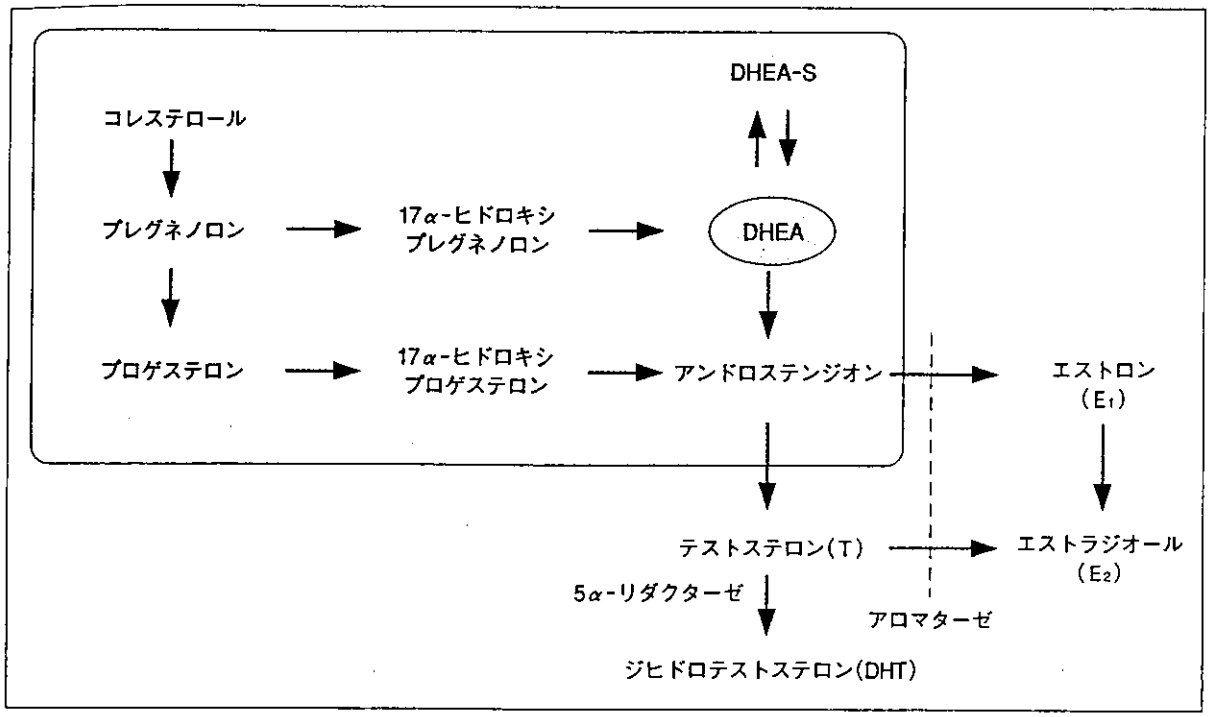



**図 24-5 DHEA の生合成経路**

DHEA はコレステロールより合成される．DHEA の一部は，末梢組織にてテストステロンやエストロゲンに変換されて作用する．

## 3. アンドロゲン補充療法

男性では閉経に相当するような急速な血中アンドロゲンの低下はみられない．加齢とともに血中アンドロゲン値は漸減するが個人差も大きい．前立腺癌の発生の問題があるため，一般に青壮年の男性性腺機能低下症を除きホルモン補充は行われない．

## 4. 副腎アンドロゲン(DHEA)の補充療法

### 1) 副腎アンドロゲンの生合成と作用機構

DHEA，DHEA-S，アンドロステンジオンは主として副腎の網状層で合成・分泌される $C_{19}$ ステロイドで副腎アンドロゲンとよばれる．血中 DHEA は DHEA-S の 0.1 ～ 1 %で，大部分は硫酸塩である DHEA-S の形で存在する．DHEA-S は血中に最も多く存在するステロイドホルモンである．しかし，そのアンドロゲン活性はテストステロンの約 5 %ときわめて弱い．

**図 24-5** に示すように DHEA は，コレステロールよりプレグネノロン，17α-ヒドロキシプロゲステロンを経て，合成される．DHEA やアンドロステンジオンの一部は末梢組織(主に脂肪組織)にてテストステロン，さらに 5α-リダクターゼによりジヒドロテストステロン(DHT)に，またアロマターゼの作用によりエストロゲンに変換されてから作用する．DHEA の特異的受容体は明らかにされていないが，DHEA の作用機構として直接作用と，テストステロンやエストラジオールへ転換されての間接作用とが考えられている(**図 24-6**)．

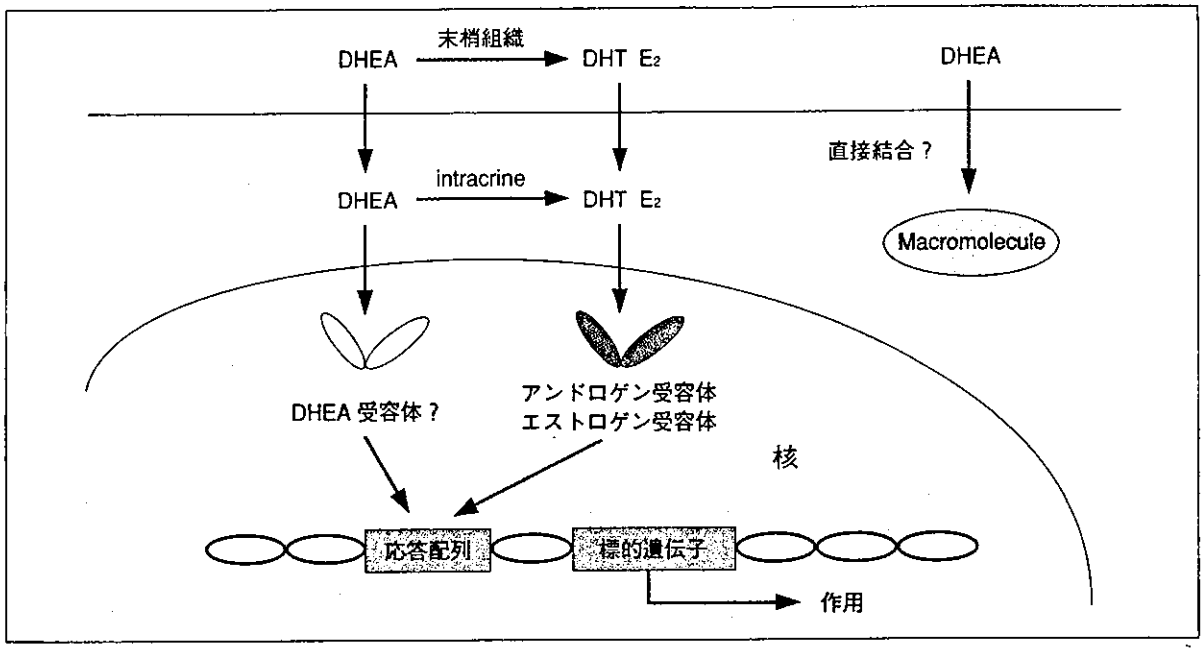
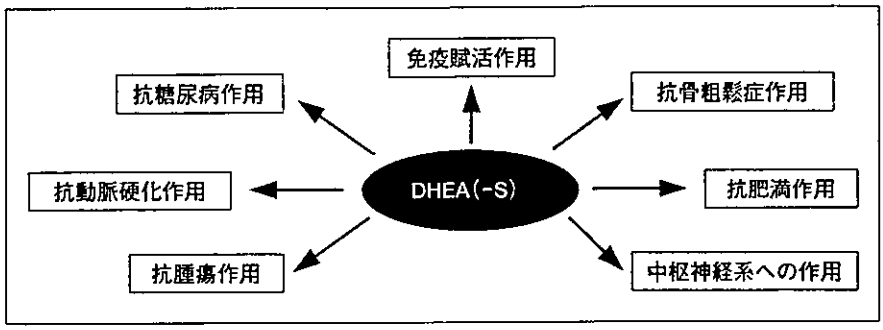



**図 24-6 DHEA の作用機構仮説**(柳瀬敏彦：Medical Tribune 35：34，2002 より)
DHEA の作用には直接作用と，テストステロンやエストラジオールへ転換されての間接作用が考えられている．

**図 24-7 DHEA-S の生物作用**
DHEA には抗糖尿病作用，抗動脈硬化作用，抗肥満作用，抗骨粗鬆症作用，免疫賦活作用など種々の作用が報告されている．




### 2) DHEA の作用と補充療法の試み

加齢により副腎アンドロゲンの分泌が低下することは以前より知られていたが，その補充療法に関しては，副腎アンドロゲンそのものが生命維持に不可欠でないことより，これまで考慮されることはなかった．近年，副腎アンドロゲン DHEA は生体にとって有益な種々の生物作用を有することが主に動物実験のレベルで証明され，特に生活習慣病の進展防止や老化制御の観点から注目されている(**図 24-7**)．

**①抗糖尿病作用**：自然発症糖尿病マウスに DHEA 投与をすると膵ランゲルハンス島の萎縮および $\beta$ 細胞の壊死を防ぎ，血糖を正常化させ，糖尿病の発症を防止できるという．また DHEA 投与により *db/db* マウスにおいて耐糖能とインスリン抵抗性の改善が報告されている．

**②抗動脈硬化作用**：家兎において DHEA 投与によりアテロームプラークが著明に縮小することが報告されている．また DHEA が培養ヒト大動脈平滑筋細胞の増殖を容量依存性に抑制し，血小板凝集を抑制する作用が報告されている．

**③抗骨粗鬆症作用**：骨芽細胞にはエストロゲン合成酵素であるアロマターゼおよびステロイド・サルファターゼが存在し，DHEA-S が DHEA，アンドロステンジオンさらにエストロンと変換されて作用する可能性がある(intracrine 作用とよばれる：**図 24-6**)．

**④免疫賦活作用**：SLE モデルマウスにおいて，DHEA は抗 DNA 抗体増加や血中 IL-2 低下を是正する．またコラーゲン誘発性関節炎に対し DHEA 反復投与は関節炎発症を遅延させ，関節炎重症度を緩和する．

米国では，1994 年より DHEA 製剤が健康補助食品(サプリメント)として認可され，容易に購入できるようになった．しかし，その純度や含量には問題もあり，副作用も明らかでない．ヒトにおける DHEA 補充の介入臨床試験が重要であり，欧米では高齢者を中心に DHEA の補充療法が試みられている．

**memo**

**intracrine 作用**：DHEA から転換されたアンドロステンジオンやテストステロンが，細胞内でアロマターゼによりエストロンやエストラジオールに転換されエストロゲンとして作用する(**図 24-6**)．このような作用機構をいい，intracrine 作用とよぶ．

### a) DHEA 投与量の検討

DHEA の至適投与量を決定するため健常女性を対象として，副腎からのアンドロゲン分泌をデキサメタゾンにより抑制した状態で DHEA を投与した場合，50 mg の DHEA を経口投与することでジヒドロテストステロンとエストロンの血中濃度を正常者と同じに維持できるという．また，性腺・副腎由来のステロイド分泌が廃絶した汎下垂体機能低下症患者を対象とした試験でも，50 mg の DHEA が適量であることが示されている．

### b) DHEA 補充の介入臨床試験

DHEA 補充の有効性を明らかにするため，信頼できる 2 重盲検試験が欧米にてなされている．Yen らのグループは高齢者を対象に 50 mg/日の DHEA を 6 ヵ月間連続投与することにより，有意の sense of well being(健康感)の改善を報告している(J Clin Endocrinol Metab 78 : 1360, 1994)．24 例のアジソン病女性に 50 mg/日の DHEA を 4 ヵ月間服用した試験では，sense of well being と sexuality の有意の改善と血中総コレステロール低下が認められている(N Engl J Med 341 : 1013, 1999)．Baulieu らによる 280 人の 60 ～ 79 歳の男女健常者を対象にした研究(DHEAge Study)では，50 mg/日 DHEA を 1 年間服用の結果，特に 70 歳以上の女性で骨密度の増加，libido の増大と発汗，色素沈着，皮膚硬化，皮脂分泌などで皮膚の改善が認められたという(PNAS 97 : 4279, 2000)．

### c) 治療薬としての期待

60 ～ 70 歳までの女性に 12 ヵ月間，連日 10 % DHEA クリームを塗布した臨床試験の報告では，子宮内膜にはエストロゲン作用を認めず，血中オステオカルシン値の上昇，尿中ヒドロキシプロリン値の低下と大腿骨骨塩量の増加を認めた．また 60 ～ 80 歳代の男女 18 名に DHEA

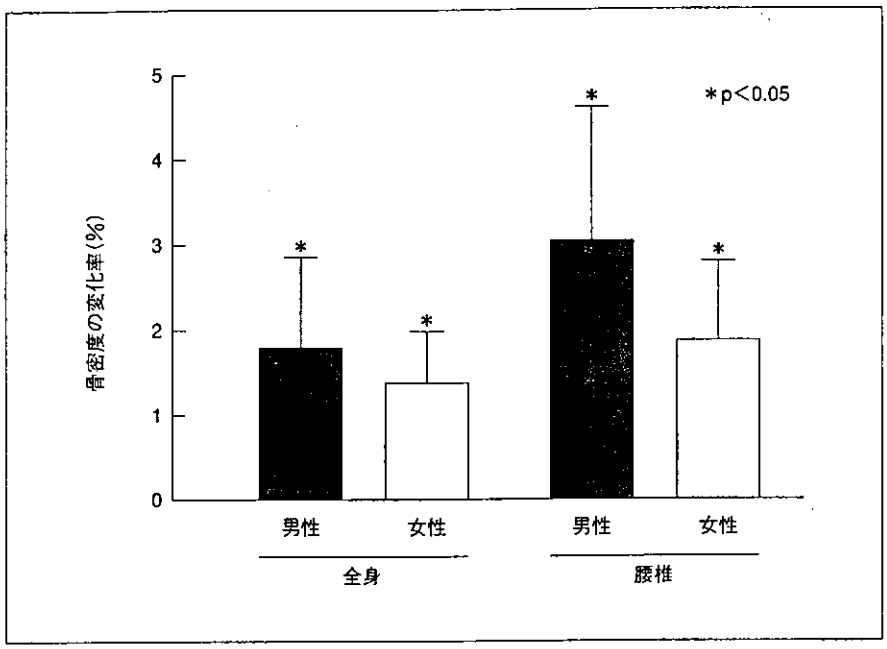



**図24-8 6ヵ月間のDHEA投与による骨密度の変化**
(Clinical Endocrinology 53：561，2000より)
60～80歳代の男女にDHEA(50mg/日)を6ヵ月投与した試験では，全身および腰椎の骨密度が有意に増加した．

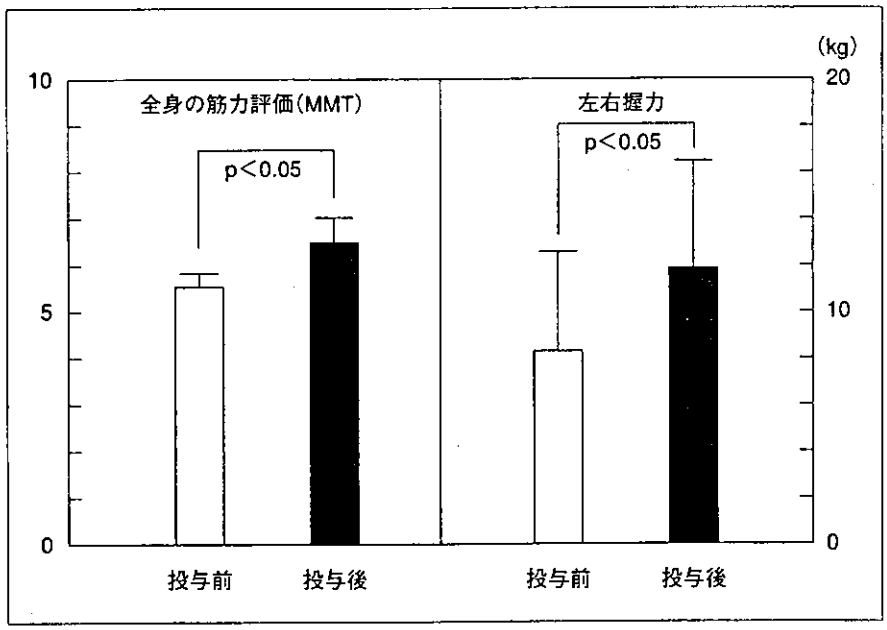



**図24-9 DHEA-S投予による myotonic dystrophy患者の筋力の改善**(Neurology 51：586，1998より)
筋強直性ジストロフィー患者に対し，DHEA-Sの静脈投与を8週間行ったところ，筋力の改善を認めた．

50 mg/日を6ヵ月内服した試験でも，全身および腰椎の骨密度が前後で有意に増加した(**図24-8**)．DHEAの骨粗鬆症治療薬としての有効性が期待されている．

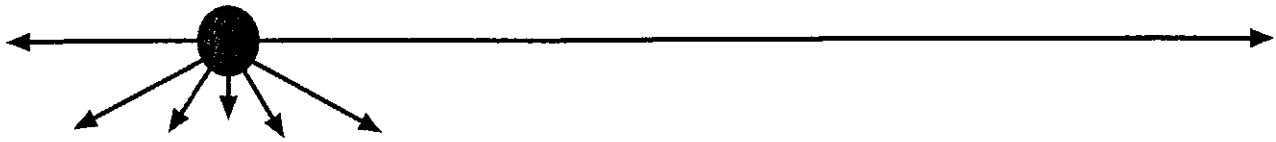
自己免疫疾患に対する効果として，50名の女性SLE(全身性エリエマトーデス)患者を対象に50～200 mgのDHEAを1年間にわたり経口投与したところ，SLEの活動性およびプレドニゾロンの必要量の減少がDHEAの投与期間を通じて観察されたという．

神経系に対する作用として，筋強直性ジストロフィー myotonic dystrophy 患者11名に対し，200 mg/日のDHEA-S静脈注射を8週間行ったところ，筋力とADLの劇的な改善を認めている(**図24-9**)．これまで有効な治療法のない疾患への新しい治療法として期待されている．

**まとめ**

- 加齢に伴うホルモンの変動に対し，閉経後の女性では女性ホルモンの補充療法が確立されている．また副腎アンドロゲンであるDHEA補充が生活習慣病予防や老化制御に有用と考えられ，臨床試験が進められており，今後の進展が期待される．

**（大中佳三，高柳涼一）**



# DHEA and the Elderly

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## Glossary

**adrenopause** Decline in adrenal production and secretion of dehydroepiandrosterone (DHEA) and DHEA sulfate after puberty.

**anti-aging hormone** A hormone, such as dehydroepiandrosterone, that improves aging-related symptoms in the elderly.

**aromatase** A cytochrome P450 enzyme that catalyzes the conversion of androgen to estrogen by aromatization.

**dehydroepiandrosterone (DHEA)** A weak androgen hormone defined chemically as Δ5-androsten-3β-ol-17-one.

**dehydroepiandrosterone sulfate (DHEAS)** Sulfate ester form of dehydroepiandrosterone.

Dehydroepiandrosterone (DHEA) and its sulfate ester, DHEA sulfate, are $C_{19}$ steroids synthesized in and secreted mostly from the adrenal gland and thus are called adrenal androgens.

## INTRODUCTION

Dehydroepiandrosterone sulfate (DHEAS) is the most abundant circulating steroid hormone in humans. Although DHEA(S) has only weak androgen activity (5% as much as testosterone), it has begun to attract great attention because of its beneficial effects as an anti-aging hormone.

## BIOSYNTHESIS AND AGE-RELATED CHANGES IN DHEA AND DHEAS

DHEA is synthesized from pregnenolone through 17α-hydroxypregnenolone by 17α-hydroxylase and 17,20-lyase. Cloning and expression analyses of the gene encoding 17α-hydroxylase revealed that both the 17α-hydroxylase and the 17,20-lyase activities are catalyzed by the same enzyme of P450c17.

Secretion of DHEA(S) from the adrenal gland alters with aging and the profile of the serum concentration of DHEA(S) shows a unique pattern (Fig. 1). The plasma concentration of DHEAS increases during adrenarche in children at the age of 6–8 years, reaches a maximal level at approximately 20 years of age, and after puberty decreases gradually throughout life. Serum DHEAS levels at 70 years of age decrease to approximately 20% of their peak values and further decrease to only 5% of peak values by the age of 85–90 years. This unique change is called adrenopause. Adrenal secretion of cortisol, which is also produced through 17α-hydroxypregnenolone in humans, essentially does not change with aging. The mechanism of dissociation between cortisol and DHEA syntheses by aging is considered to be due to the decreased activity of 17,20-lyase relative to 17α-hydroxylase activity in P450c17. It has been reported that a relative decrease in the 17,20-lyase activity results from an age-dependent decrease in enhancers of the 17,20-lyase activity, such as cytochrome *b5* and P450 reductase, and also by inactivation of 17,20-lyase by adrenal lipid peroxidation. Age-dependent atrophy of the zona reticularis, which is thought to be the principal site of DHEAS secretion, has also been reported.

The decrease in adrenal secretion of DHEA(S) after puberty was observed only in primates such as humans and rhesus monkeys. In rhesus monkeys, the serum level of DHEAS declines two times as rapidly as in humans. However, dietary caloric restriction in rhesus monkeys elongates the life span accompanied by lower body temperature, lower levels of plasma insulin, and a slow rate of decline in serum DHEAS. A longitudinal study of the survival rate of humans in Baltimore, Maryland, also showed that men with lower body temperature and plasma insulin and those maintaining higher serum DHEAS levels exhibit longer survival than the respective counterparts.

*Encyclopedia of Endocrine Diseases, Volume 1.* 

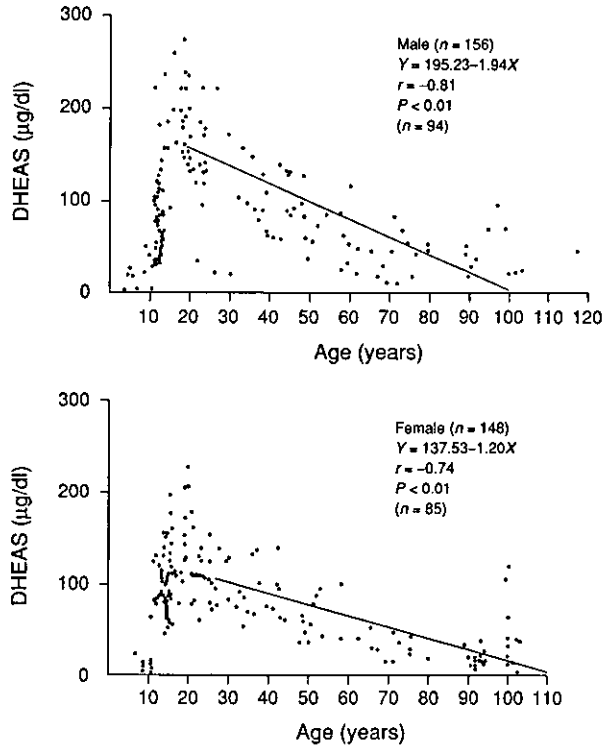


**Figure 1** Age-related change in plasma DHEAS in the male and female.

## BIOLOGICAL EFFECTS AND MECHANISM OF ACTION OF DHEA

The mechanism of action of DHEA(S) remains unclear, but three possible mechanisms have been hypothesized. The first possible mechanism is a direct action through the specific receptor. Although specific binding sites for DHEA have been reported in various cells and tissues, the receptor has not been successfully cloned as yet. The second possible mechanism is an indirect action, in which DHEA is converted to testosterone, or is further converted to estrogen by aromatase, in peripheral tissues and acts through the androgen receptor or the estrogen receptor. Thus, DHEA can be converted to active sex steroids and function in the same cell. This mechanism is called "intracrine action." The third possibility is that the hydrophobic DHEA molecule may alter cell function by interacting with certain macromolecules, such as enzymes.

Experimental studies have demonstrated that DHEA(S) has various beneficial effects including an anti-diabetic effect, prevention of atherosclerosis,

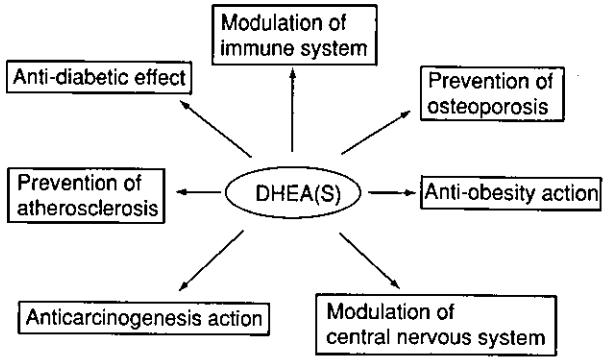


**Figure 2** Beneficial effects of DHEA(S).

anti-obesity action, prevention of osteoporosis, and modulation of the immune system (Fig. 2).

### Anti-diabetic Effect

It is reported that administration of DHEA improved insulin resistance by suppression of the increased activities of glucose-6-phosphatase and fructose-1,6-bisphosphatase in a non-insulin-dependent diabetes model, db/db mice. DHEA is also reported to improve insulin resistance observed in rats and decrease the serum level of tumor necrosis factor-*a* in obese Zucker rats. DHEA increases glucose uptake *in vitro* through the activation of protein kinase C or phosphatidylinositol 3-kinase. In human studies, the serum DHEAS level is reported to be lower in non-insulin-dependent diabetic patients than in age-matched normal male subjects.

### Anti-obesity Action

An anti-obesity effect of DHEA has been observed in animal studies and may be related to several mechanisms including decreased lipogenesis and increased thermogenesis. In humans, the plasma concentration of DHEA decreases inversely with body mass index.

### Prevention of Atherosclerosis

There are many retrospective and cross-sectional human studies demonstrating reciprocal relationships between the serum concentration of DHEA(S) and the incidence of ischemic heart disease. Animal studies also showed that the administration of DHEA

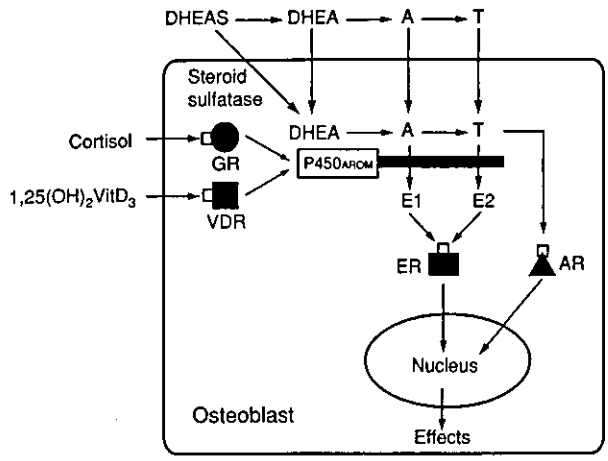


Figure 3 Schematic representation of intracrine action of DHEA in the osteoblast. GR, glucocorticoid receptor; VDR, vitamin D receptor; ER, estrogen receptor; AR, androgen receptor; A, androstenedione; T, testosterone; E1, estrone; E2, estradiol; $1,25(OH)_2VitD_3$, 1,25-dihydroxyvitamin $D_3$; $P450_{AROM}$, cytochrome P450 aromatase.

suppressed atherosclerosis induced by intimal injury or a high-cholesterol diet. It has been reported that DHEA suppressed the accumulation of cholesterol esters induced by the addition of acetylated low-density lipoprotein in cultured macrophage cells.

### Prevention of Osteoporosis

Postmenopausal osteoporosis is a widespread clinical problem that correlates with low circulating levels of sex steroid hormones. A significant positive correlation between bone mineral density and the serum DHEAS level, but not the serum estradiol level, was observed in postmenopausal women, suggesting a possible role for DHEA(S) in preventing osteoporosis. It has been demonstrated that primary cultured human osteoblasts have aromatase activity, which is enhanced by dexamethasone and 1,25-hydroxyvitamin $D_3$ (Fig. 3). These results suggest that osteoblasts may utilize the circulating DHEA(S) and convert it to estrogen by aromatase to maintain bone mass after menopause.

## REPLACEMENT THERAPY OF DHEA IN HUMANS

The replacement of DHEA has attracted attention in view of both the prevention of age-related diseases and the control of aging. DHEA is commercially available as a food supplement in the United States, but the purity and content of that DHEA are not clearly specified. Clinical intervention trials of DHEA replacement are essential for establishing the significance of that therapy. Results from three randomized placebo-controlled studies support the concept that oral replacement of DHEA has beneficial effects.

Six months of daily replacement of DHEA (50 mg/day) in healthy adults (13 men and 17 women) restored the level of DHEA and DHEAS to the high levels observed in young people and caused an increase in the serum concentrations of androgen and insulin-like growth factor-I. This result is associated with a remarkable increase in perceived physical and psychological well-being in both sexes without an effect on libido.

The administration of DHEA (50 mg/day) in 24 female patients with adrenal insufficiency normalized serum concentrations of DHEA, DHEAS, androstenedione, and testosterone. DHEA significantly improved overall well-being as well as scores for depression and anxiety, the frequency of sexual thoughts, sexual interests, and satisfaction of both the mental and physical aspects of sexuality.

Two hundred eighty healthy men and women (60–79 years old) were given DHEA (50 mg/day) or placebo orally for 1 year in a double-blind, placebo-controlled study (DHEAge Study). DHEA administration reestablished a "young" concentration of DHEAS and a small increase in testosterone and estradiol with no adverse side effects. A significant increase in most libido parameters was observed in older women. An improvement in skin status in terms of hydration, epidermal thickness, sebum production, and pigmentation was also noted, particularly in women. Bone turnover was improved in women over 70 years of age as assessed by bone mineral density and bone resorption markers.

## CLINICAL TRIAL FOR THE TREATMENT OF VARIOUS DISEASES

In a study of 14 postmenopausal women, 12 months of daily administration of DHEA via 10% cream caused a significant increase in bone mineral density accompanied by a 2.1-fold increase in the plasma osteocalcin level with no adverse side effects. Oral replacement of DHEA (50 mg/day) in 18 elderly people also increased bone mineral density and decreased fat mass.

The intravenous administration of DHEAS (200 mg/day for 8 weeks) in 11 patients with myotonic dystrophy led to an improvement in the activities of

daily living, an increase in muscle strength, and a decrease in myotonia.

Oral administration of DHEA (50–200 mg/day for 1 year) in 50 female patients with systemic lupus erythematosus (SLE) induced a decrease in the activity of SLE and led to a decrease in the requisite dose of prednisolone.

## CONCLUSION

DHEA is theorized to act as an anti-aging hormone against age-related diseases. However, it is not known whether or not the increase in sex steroid levels in long-term DHEA replacement is safe with regard to the development of steroid-dependent cancers, such as ovarian and prostate cancers. It is necessary to establish the clinical evidence for DHEA replacement therapy in the elderly by conducting prospective long-term randomized studies.

## See Also the Following Articles

Adrenal Androgens • Aging and Longevity of Human Populations • Aging: Muscle • Alzheimer's Disease and Hormones • Neuroendocrine System and Aging • Osteoporosis in Older Men • Osteoporosis in Older Women

## Further Reading

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Naotaka Hamasaki

# The analysis of biocatalysts

*The development of clinical enzymology*

*Micrograph of crystals of the digestive enzyme trypsin, taken in polarised light.*

Until the nineteenth century it was widely believed that a special life force or *élan vital,* operating only in higher living beings, was required to convert inorganic material into organic compounds. For the 'vitalists' it was simply inconceivable that biological functions might one day be explained by chemistry. By the end of the nineteenth century, however, opposition to vitalism was growing, and with it the tendency to explain the phenomena of life in terms of physicochemical processes.[1] This was also the century that saw the first discovery of an enzyme, although enzymatic phenomena had in fact been observed long before. Some 200 years earlier, for example, it had been noted that meat liquefied in the gastric juice of hawks, which therefore had to contain some protein-dissolving substance. Anselme Payen (1795–1871) and Jean-François Persoz (1805–1868) were probably the first to isolate an enzyme of plant origin. They treated an aqueous extract of malt with ethanol and precipitated a heat-labile substance which promoted the hydrolysis of starch. They called the fraction thus extracted 'diastase', deriving the term from the Greek word for 'separation', as this enzyme separates sugar components from starch. It is now recognised that Payen and Persoz's diastase was in fact an impure preparation of amylase.[2] The next enzyme to be partially purified was of animal origin. Theodor Schwann (1810–1882), the German physician and biochemist to whom we owe ground-breaking descriptions of cell structure and metabolism, isolated a substance from gastric juice that cleaved and dissolved protein. He called it 'pepsin'.

Pepsin was clearly one of those long-sought substances that initiated and accelerated changes in organic compounds. As yet they had no name, and

it was only possible to speculate about their effects. In 1836, the same year that Schwann described pepsin, the Swedish naturalist Baron Jöns Jacob Berzelius (1779–1848) wrote:

> We have good reason to suppose that, in living animals and plants, thousands of catalytic processes take place between tissues and fluids and bring forth the multitude of heterogeneous decompositions that we shall in future perhaps discover in the catalytic power of the organic tissue of which the organs of the living body consist.[3]

It was these initial observations of enzymatic activity that paved the way for the notion of 'catalysis'.[4] Between 1849 and 1877 other enzymes were identified, such as pancreatic lipase[5] (discovered by Claude Bernard, 1813–1878) and invertase in yeast[6] (Marcelin Berthelot, 1827–1907).

At the time of these early discoveries it was not yet clear that yeasts were unicellular organisms. That they were alive and responsible for fermentation was demonstrated independently, and almost simultaneously, in 1837 by Charles Cagniard-Latour (1777–1859), Schwann and Friedrich Traugott Kützing (1807–1893). As so often in the history of science, these convergent discoveries were due to improvements in instrumentation – in this case, the development of microscopes with achromatic lenses.[7] Cagniard-Latour noted that the cells of brewer's yeast consisted of a mass of small round units capable of reproduction and were not simply an organic or chemical substance. The idea that fermentation was caused by living organisms was confirmed and pursued further by Louis Pasteur (1822–1895).[8] He also coined the noun 'ferment' for the catalysts involved. While Pasteur considered the processes that occur during fermentation to be essential physiological activities of certain microorganisms, contemporary chemists – notably Justus [von] Liebig (1803–1873) – advocated a purely chemical theory of fermentation. A distinction was drawn between 'organised ferments' (most famously espoused by Pasteur), which were believed to be present in or on the surface of living cells, and 'unorganised ferments' such as diastase and pepsin, the activity of which was clearly not associated with microorganisms (the view championed by Liebig).[9] The dispute between Pasteur and Liebig was not settled until 1897, when two brothers, Hans Ernst August Buchner (1850–1902) and Eduard Buchner (1860–1917), obtained a cell-free yeast extract that converted sugar to alcohol.[10,11] The term 'enzyme' (from the Greek for 'in yeast') as a name for biocatalysts, or ferments, was introduced by Friedrich Wilhelm Kühne (1837–1900), and led naturally to the idea of a science of enzymes, i.e. enzymology. However, the fact that enzymes are proteins was not recognised until the late 1920s. When James Batcheller Sumner (1887–1955) crystallised urease in 1926,[12] many argued that the enzyme was simply an impurity adsorbed onto or occluded within the protein crystals. But in the early 1930s John H. Northrop (1891–1987) and co-workers crystallised pepsin, trypsin and chymotrypsin, and conclusively demonstrated that the protein crystals were pure enzymes. By 1943 about 25 enzymes had been crystallised, and enzymology was firmly established as a scientific discipline.

*The German biochemist* **Eduard Buchner** *(1860–1917), working with his brother Hans Ernst August Buchner (1850–1902), showed that a cell-free extract of yeast was capable of fermenting sugar to alcohol. After observing that gas formed when a concentrated cane sugar solution was added as a preservative to yeast juice, he realised that a fermentation reaction had occurred which could help to settle the dispute between Pasteur and Liebig. On 11 January 1897, Buchner's six-page paper on 'Alcoholic fermentation without yeast cells' was read at a meeting of the German Chemical Society; the impact of his finding was similar to that of the discovery of X rays by Wilhelm Konrad Röntgen (1845–1923) two years earlier. Buchner showed that substances can have catalytic properties which do not necessarily depend on cellular structures. Ten years later, in 1907, he received the Nobel Prize in Chemistry for validating the concept of enzymes and establishing its general applicability to biochemical catalysts acting inside and outside cells.[35] Although he was 54 when the First World War broke out, he volunteered for active service and died in 1917 from a shrapnel wound suffered in action at the front in Romania.*

## Enzymatic analysis and clinical enzymology

In the early 1900s the events involved in enzymatic reactions were studied more closely, and the concept of kinetic analysis was established by Leonor Michaelis (1875–1949) and Maude Leonora Menten (1879–1960) in Berlin and George Edward Briggs (1893–1985) and John Burdon Sanderson Haldane (1892–1964) in Britain. In 1913 Michaelis and Menten rediscovered the equation derived by Victor Henri (1872–1940).[13] The Henri-Michaelis-Menten equation, which is based on simple principles of chemical equilibrium,[14] is a measure of the affinity between enzymes and substrates. In 1925 Briggs and Haldane introduced the concept of steady state into enzyme kinetics.[15] In the 1940s and 1950s hundreds of new enzymes were discovered, many of which were purified to homogeneity and crystallised. Key metabolic pathways were elucidated, and biochemists began to focus on the mechanisms of

**Leonor Michaelis** *(1875–1949), a biochemist, was in charge of the bacteriological laboratory at Urban Hospital in Berlin. Together with Maude Menten (1879–1960) – a Canadian physician and biochemist, who was a visiting researcher at the University of Berlin in 1912 – he developed the famous Michaelis-Menten equation. This enabled scientists for the first time to describe biochemical reactions mathematically. Michaelis and Menten's findings still serve as a basis for describing the catalytic activity of proteins. Their work also helped pave the way for the development of industrial biotechnology.*[37]

## Enzymes and substrates

Enzymes are large proteins which play an essential role as biocatalysts in a multitude of metabolic reactions. Intensive study in the early part of the 20th century revealed them to be highly complex molecules of varying molecular weight, consisting of long chains of 20 amino acid building blocks whose arrangement (sequence) differs for each enzyme. These chains have to be folded into specific three-dimensional shapes for enzymes to function as catalysts.

The substances converted by enzymes into other compounds are referred to in scientific parlance as 'substrates'. Conversion occurs when a substrate binds to a special region of an enzyme, known as its 'active site'. The enzyme itself remains unchanged by this reaction. Each substrate fits the active site of a specific enzyme as neatly as a key fits the lock it was made for – a property known as 'substrate specificity'.

Enzymes are denoted by the names of their substrates, followed by '-ase'. The enzyme that cleaves maltose (malt sugar), for example, is called 'maltase'. The vast number of enzymes which have been discovered to date are divided into six groups according to the reactions they catalyse. Hydrolases, for example, are a group of enzymes that cleave certain chemical bonds, whereas ligases catalyse the formation of new chemical bonds.

enzyme activity and regulation. Reactions catalysed by enzymes were described in the equations of enzyme kinetics. During the same period Otto Heinrich Warburg (1883–1970) developed a method of 'enzymatic analysis' based on the absorption of light by the pyridine coenzymes NADH and NADPH at a wavelength of 340 nanometres.[16] The introduction of the spectrophotometer by Arnold Orville Beckman (b. 1900) and Carl Zeiss (1816–1888), and the commercial production of purified metabolic enzymes by Boehringer Mannheim, established enzymatic analysis in the laboratory.

During this period biochemists were also attempting to standardise a variety of measurement systems, nomenclatures and units for properties such as molecular weight. Amidst this welter of information, Hans Ulrich Bergmeyer (1920–1999) resolved to publish a handbook that would make enzymatic analysis practicable even for non-experts. Recognising the biological significance of enzyme reactions, Bergmeyer was convinced that progress in biology would depend on the availability of reliable methods of enzymatic measurement. His *Methods of Enzymatic Analysis*, published in 1963, was intended not as a theoretical treatise but as an introduction to the fundamentals of measurement. This handbook has introduced generations of clinical chemists to the principles and methods of enzymatic analysis.[17]

## Clinical enzymology and laboratory medicine

The foundations of clinical enzymology were laid in the early 1900s, when serum amylase (AMY) was first measured in patients with pancreatic disease.[18] At the end of the 1930s (acid and alkaline) phosphatases and cholinesterase were found to be clinically relevant in prostate cancer and liver disease.[19,20,21] They were joined approximately 20 years later, in the mid-1950s, by alanine aminotransferase (ALT), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) – markers of liver disease and myocardial infarction, and mainstays of contemporary clinical enzymology.[22,23] These were soon followed by the contribution of creatine kinase (CK) and γ-glutamyltransferase (γ-GT) to the understanding and management of skeletal muscle disorders, myocardial infarction and liver disease. Measurements of enzyme activity in body fluids provided such useful diagnostic information that they became an increasingly important adjunct to routine clinical examination.[24,25]

The adoption of enzymatic analysis in routine laboratory settings was facilitated by the develop-

ment of excellent assay methods and by remarkable innovations and improvements in assay equipment. The growth and progress of clinical enzymology may thus be regarded as a product of collaboration between industry and academia.

Apart from its importance in the clinical laboratory, enzymology occupied centre stage in biochemistry and the life sciences until about 1970. By that time most of the body's metabolic pathways and key metabolic enzymes had been investigated in detail. Today the cellular metabolic reactions once viewed as 'vital phenomena' can be reproduced in the test tube (in vitro) and elucidated without invoking any mysterious life force. Moreover, enzyme kineticists are now able to go beyond qualitative 'all-or-nothing' descriptions and characterise biological phenomena in the quantitative language of mathematics and trace metabolic changes to changes in the balance between enzyme systems.[26,27] Quantitative analysis is an important tool for interpreting metabolic phenomena generally, and not only the role played by enzymes. Replace the first word in 'enzyme kinetics' with 'protein', and you have an effective quantitative/kinetic approach for analysing biological processes and events based on protein-protein interactions. Signal transduction events in cells, for example, cannot be precisely elucidated except by means of quantitative/kinetic analysis.[28] Thanks to advances in the quantitative analysis of enzyme reactions, it is now possible to explain 'vital phenomena' on the basis of structures at the amino acid side chain level ('structural biology'). Viewed from this perspective, enzymology is at the origin of today's life sciences.

Since its inception in the early 1900s, the mission of clinical enzymology has been to apply research findings in the diagnosis of disease, essentially by measuring enzyme activity in serum or plasma. In almost every case – the exceptions being blood coagulation factors and enzymes involved in lipid metabolism – the enzymes of interest are released into the circulation from damaged cells or tissues, whose identification thus becomes of critical importance. The main tools used by enzymologists for this purpose have been isoenzymes, or isozymes, and mapping techniques which have assigned these isozymes to tissues, cells and even cell components. Isozymes – the concept dates from 1959[29] – are enzyme variants present in the same organism and catalysing the same reaction, but differing in their molecular protein structure and physicochemical properties. Analysis of the primary structure[30] of released enzymes also sheds light on the etiology and pathology of enzyme abnormalities. Such analyses were once confined to specialised facilities, but advances in genetic engineering have made them routine procedures in clinical laboratories. Most of the enzymes currently investigated have been known since the early days of clinical enzymology. The prescience and foresight of the pioneers in this field beggar belief.

*From 1954 until his retirement in 1985,* **Hans Ulrich Bergmeyer** *(1920–1999) worked as a chemist and manager at Boehringer Mannheim in Tutzing, near Starnberg Lake in Upper Bavaria. His initial task was to determine the activity of the enzyme extracts which the company produced for therapeutic use. For this purpose he developed new methods which he subsequently systematised in what was to become a standard reference work. Bergmeyer's* Methods of Enzymatic Analysis, *which has gone through more than ten editions since it was first published in 1963, contains hundreds of detailed analytical methods. The translation of his findings into commercial products, including enzymes and substrates for research and enzyme-based diagnostic tests, established him as a pioneer of modern diagnostics.*

While clinical enzymology's mission remains unchanged, the automated analysers introduced over the last few decades have dramatically improved the measurement of circulating enzymes. Not only have measurements of enzyme activity become significantly more accurate, but automation has also reduced the time and sample sizes required for testing. Results are also available faster because today's systems are able to analyse large numbers of samples simultaneously. Moreover, these revolutionary performance characteristics are now packaged in analytical instruments so compact and simple to use that they can provide precise, accurate and rapid enzyme data at the bedside.

## Measuring released enzymes

As it circulates through the body, blood not only supplies oxygen to cells and tissues, but also transports waste generated at various sites to the lungs and kidneys for elimination. Thus enzymes released

from injured cells or tissues enter the circulation, where – unless they are inactivated – their increased presence indicates damage to their source organ. Enzymes commonly measured for their clinical implications include the transaminases (AST, ALT), LDH, γ-GT, alkaline phosphatase (ALP), CK, AMY, cholinesterase (ChE) and lipase. Since they catalyse major metabolic reactions, many of them occur in organs. If an organ containing significant amounts of a given enzyme is damaged, it will release greater amounts of that enzyme into the blood. The elevated enzyme level thus becomes a sensitive marker of organ damage. Typical examples include CK and ALT: an increase in blood CK levels indicates muscle injury, while elevated blood ALT levels are almost certainly a sign of liver damage. By contrast, the distribution of LDH is less organ-specific, making it difficult to identify the injured organ on the basis of increased blood LDH levels. However, analysis of LDH isozymes is effective for this purpose. Similarly, CK isozyme analysis can distinguish myocardial damage from skeletal muscle injury. Enzyme kinetics can also be recruited to identify the site or severity of damage, e. g. by measuring the slope of the increase in levels of an enzyme or the time of the increase. It is generally thought that enzymes are released into the bloodstream through damaged cell membranes, but this has not been experimentally proven. For example, although glucose-6-phosphatase is specific to hepatocytes, its clinical usefulness in diagnosing liver damage has proved to be no greater than that of ALT. The presumed reason is that glucose-6-phosphatase, which occurs in the membrane of the endoplasmic reticulum, is not released from the cell as readily as ALT or LDH, which are present in the cytoplasm. But, again, experimental evidence to support this hypothesis is lacking.

### Enzyme markers in clinical diagnosis

| Enzyme | Diagnosis | First reported |
|---|---|---|
| Amylase | Acute pancreatitis | 1910 |
| Acid phosphatase | Prostate cancer | 1938 |
| Cholinesterase | Liver disease | 1938 |
| Alkaline phosphatase | Liver disease | 1940 |
| Aspartate aminotransferase | Myocardial infarction | 1954 |
| Aspartate aminotransferase | Hepatitis | 1956 |
| Alanine aminotransferase | Liver disease | 1956 |
| Lactate dehydrogenase | Myocardial infarction | 1956 |
| Glutamate dehydrogenase | Leukemia, cancer | 1956 |
| Lactate dehydrogenase | Cancer | 1957 |
| Lactate dehydrogenase | Muscle disease | 1957 |
| Creatine kinase | Muscle disease | 1959 |
| Creatine kinase | Myocardial infarction | 1960 |
| γ-Glutamyltransferase | Liver disease | 1960 |

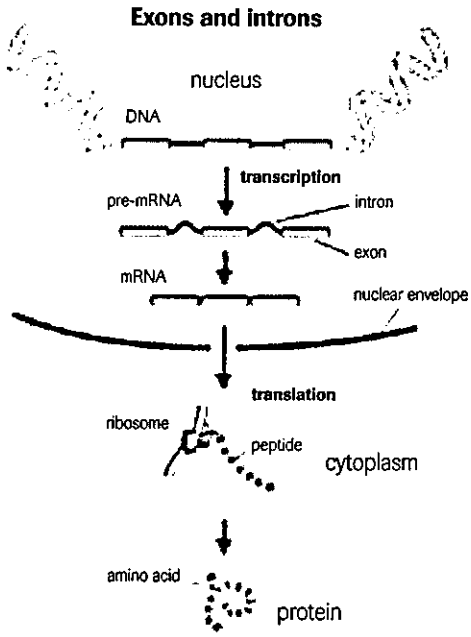
### Isozymes

Since the same enzymes may occur in many organs, an increase in the activity of a particular enzyme in a body fluid does not always identify the damaged organ. This is where isozyme analysis comes to the rescue. LDH and CK are prime examples of enzymes routinely subjected to isozyme analysis. In the case of LDH, five isozymes have been identified: H4, H3M1, H2M2, H1M3 and M4 ('H' denoting a subunit expressed primarily in heart tissue, and 'M' a subunit expressed primarily in skeletal muscle). The gene for the H subunit is located on chromosome 12 (12p12.2-p12.1) and that for the M subunit on chromosome 11 (11p15.1-p14).

The IUPAC-IUB[31] Joint Commission on Biochemical Nomenclature recommends restricting the term 'isozymes' to multiple forms of enzymes arising from genetically determined differences in primary structure (e. g. proteins encoded by different genes) heteropolymers with these proteins as subunits (hybrids) and a group of polymorphic molecules that have clearly resulted from mutations. However, in the clinical laboratory setting, enzymes resulting from post-translational protein modification are also recognised as isoforms. The techniques used for isozyme analysis include electrophoresis, ion exchange column chromatography and immunological methods. Electrophoresis separates isozyme fractions according to their charge characteristics. Immunological methods use specific antibodies to distinguish between isozymes.

### Measuring enzyme activity

Virtually every clinical laboratory now uses automated analysers to measure enzyme activities, and as a result there have been significant gains in the precision of such measurements. Also, at the level of individual laboratories, precision has been greatly enhanced by the application of internal quality controls. However, standardisation of test results between laboratories has emerged as a major problem. A satisfactory solution is urgently required, particularly given the move towards electronic patient records and the related prospect of more and more strictly regimented standards of care. Where quantities of a substance can be expressed as concentra-




### Genetic polymorphisms and abnormal proteins

The gene regions that code for proteins are known as exons, and their adjacent non-coding regions as introns. During the editing process that results in a mature ribonucleic acid molecule (messenger RNA, or mRNA), the introns are removed, leaving behind the sequences that contain instructions for protein synthesis. If a variation associated with substitution of an amino acid in a protein is detected in an exon region, this suggests the presence of a gene polymorphism, which may or may not result in expression of an abnormal protein. To establish whether a variation is a polymorphism, the gene of interest can then be analysed in 50–100 individuals to determine the frequency of the variation. An enzyme molecule can be confirmed as abnormal by introducing the abnormal gene into cell cultures (transfection) and then examining the enzyme's specific activity.

*Introns are removed during RNA maturation. The resulting strand of mRNA carries the instructions for constructing a protein from the cell nucleus to the ribosomes in the cytoplasm, where protein biosynthesis takes place.*

tions (as with electrolytes and glucose), measurements can be standardised relatively easily with the aid of standard reference materials. However, this is not readily applicable to enzyme measurements. The activities measured for one and the same enzyme preparation vary with the conditions of measurement. In addition, the commercial reagents used in laboratories are prepared for measurements based on different principles and performed under different conditions, which inevitably gives rise to discrepancies in the results obtained by different laboratories. But if that is the case, is inter-laboratory variation something we can reasonably hope to eliminate? Is standardisation a realistic aim in clinical enzymology? The answer is 'Yes', for the following reasons.

Although the term 'true value' surfaces from time to time in discussions about measuring enzyme activity, there is no such beast: all values are 'apparent'. Once this basic principle is grasped, the standardisation problem looks less intractable. Ultimately, it is impossible to evaluate the accuracy of measurements of enzyme activity. However, the values obtained using one assay can be correlated with those obtained using another. A method that unequivocally validates the specific activity of an enzyme with a high degree of precision should be selected as the reference method. Practical standardisation of measured enzyme activity values is not difficult provided the method chosen gives a high specific activity and is precise, and there is a reference enzyme preparation. Progress on this front has recently been facilitated by the advent of commercial enzyme reference materials (ERMs).

### Lipid metabolism and blood clotting

Research into lipid metabolism has made remarkable progress in recent times, a fact reflected by increased clinical testing for lipoprotein fractions and other components of lipid metabolism. This development has been paralleled by the increasingly frequent measurement of enzyme activities relevant to lipid metabolism, e.g. of lipoprotein lipase (LPL). Cholesteryl ester transfer protein (CETP) is also measured, although it is not an enzyme. Until recently, cholesterol levels in lipoproteins rather than total serum cholesterol were considered an important atherogenic factor, with lower concentrations of low-density lipoprotein (LDL) and higher concentrations of high-density lipoprotein (HDL) cholesterol being thought to lower the risk of atherosclerosis.[32] However, improved understanding of substances such as hepatic triglyceride lipase (HTGL) has sparked a growing controversy over the protection afforded by high HDL levels. Certainly it would be premature to base arguments about the

*Computer image of lipase, an important enzyme in lipid metabolism.*

promotion or prevention of atherosclerosis simply on measurements of HDL cholesterol.

While thrombosis among Japanese was once thought to be less common than among Europeans or Americans, its incidence in Japan is now recognised as relatively high. Thrombosis is a multifactorial disorder caused by a combination of lifestyle and environmental factors. Moreover, low anticoagulation factor activity has been found in many people with an increased tendency to thrombosis.[33] There is growing recognition of the importance of measuring the activity of anticoagulation factors such as protein C, protein S and antitrypsin, and of fibrinolytic factors such as plasminogen and plasmin inhibitor. Although they do not all have enzyme activity, some are proteinases or enzyme activators or inhibitors.

These enzymes and factors involved in lipid metabolism and blood coagulation have physiological functions in the circulation. Changes in their activity reflect direct physiological effects on metabolic systems and carry greater clinical significance than the levels of disease-marker enzymes released into the circulation by damaged tissue. However, no basis has yet been established for their investigation in the clinical laboratory. Whether they eventually become incorporated into routine tests depends on the development of suitable assay methods.

## Abnormal enzyme molecules

When a clinical sample shows abnormally low or high levels of enzyme activity that cannot be attributed to the patient's disease, immunoglobulin[34] binding or a molecular abnormality may be the cause. Immunoglobulin-bound enzymes can be detected by a combination of isozyme analysis and immunological staining. The presence of abnormal enzyme molecules is indicated by a discrepancy between the activity and quantity (concentration) of an enzyme. Until a few decades ago, when genetic engineering was still in its infancy, identifying abnormal molecules involved the use of cumbersome techniques of protein chemistry. Today, however, identification is relatively easy. After obtaining the patient's informed consent, a blood sample is taken, and deoxyribonucleic acid (DNA) is extracted from the white blood cells. This genetic material is then amplified (copied many times) using the polymerase chain reaction (PCR) technique, and the DNA sequence is examined for abnormal sites.

## Prospects

Clinical enzymology owes its current standing to a series of tremendous advances that began in the 1950s. Its clinical utility is beyond question, and its techniques are an indispensable part of modern diagnostic practice. Yet at the same time it could be argued that few developments of note have occurred since the 1980s. Be that as it may, the techniques used in clinical enzymology to detect disease in microlitres of blood will continue to play an important role in the future. Enzymes with physiological functions in blood and those released into the circulation by damaged tissue will remain key parameters in laboratory diagnostics. The next leaps forward are likely to occur when pathological changes can be assessed by testing for circulating levels of various proteins involved in intracellular signal transduction as well as for enzymes, tumour markers and cytokines.

Beckoning on the more distant horizon is a new era in measurement technology. Nothing would be more desirable than to be able to perform clinical chemistry tests non-invasively. I have a vision of the future in which hospitals will be equipped with walk-through diagnostic systems capable of doing a laboratory workup of patients as soon as they arrive. For patients this will mean that some of the familiar 'trials' of the examining room will be replaced by a procedure as simple and non-invasive as stepping through a metal detector gate at the airport.[35]

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# Glucocorticoid enhances the expression of dickkopf-1 in human osteoblasts: novel mechanism of glucocorticoid-induced osteoporosis

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**Abstract**

To clarify the underlying mechanism of glucocorticoid-induced osteoporosis, we investigated the effect of glucocorticoid on the expression of dickkopf-1 (Dkk-1), an antagonist of Wnt signaling, in primary cultured human osteoblasts. Dexamethasone markedly induced the expression of mRNA for Dkk-1 in a dose- and time-dependent manner. The expression of Kremen1, a receptor for Dkk, did not change by the treatment with dexamethasone, while that of low-density lipoprotein receptor-related protein 5 (LRP5), a Wnt coreceptor, slightly decreased by the treatment with dexamethasone. Dexamethasone increased the transcriptional activity of the Dkk-1 gene promoter in human osteoblasts. Serial deletion and mutation analyses of the Dkk-1 promoter showed that one putative glucocorticoid responsive element-like sequence located from −788 to −774 bp is essential for the enhancement of the Dkk-1 promoter activity by dexamethasone in human osteoblasts. Since the Wnt signal is now recognized as a crucial regulator for bone formation, the Dkk-1 enhanced by glucocorticoid may inhibit the Wnt signal in osteoblasts, which may be involved in the pathogenesis of glucocorticoid-induced osteoporosis.


*Keywords:* Glucocorticoid; Wnt; Dickkopf-1; Osteoblast; Osteoporosis

The Wnt family is a secreted glycoprotein that participates in morphogenesis, determination of cell polarity, and regulation of cell proliferation and differentiation during embryogenesis [1,2]. The Wnt proteins bind to frizzled family of seven transmembrane domain receptor and its coreceptor low-density lipoprotein receptor-related protein 5 (LRP5) and LRP6 [3]. Canonical Wnts inactivate glycogen synthase kinase-3β (GSK-3β) and inhibit phosphorylation and consequential degradation of intracellular β-catenin [1–3]. Accumulated β-catenin translocates into the nucleus and activates target genes by complex with transcription factors of the T-cell factor (Tcf)/lymphoid enhancer factor (Lef) family [1–3]. The Wnt signal is regulated by two classes of extracellular antagonists [4]. Secreted frizzled-related protein (sFRP), Cerberus, and Wnt inhibitory factor-1 (WIF-1) are inhibitors that bind Wnts and restrict the Wnt function. Dickkopf (Dkk) family is another class of secreted Wnt antagonist. Dkk interacts with the Wnt coreceptor LRP5 and LRP6, and inhibits Wnt signaling by disturbing the binding of LRP5/6 to the Wnt/frizzled ligand–receptor complex [5].

Recent analyses of patients with the LRP5 gene mutation and LRP5 knockout mice revealed that LRP5 plays pivotal roles in bone metabolism [6–8]. It is reported that the missense mutations of LRP5 gene cause osteoporosis-pseudoglioma syndrome in which bone and eyes are abnormally developed [6]. LRP5 knockout mice also showed similar phenotype in which low bone density, decreased osteoblast proliferation that is independent of *Runx2/Cbfa1*, and abnormal eye development were observed [7]. On the other hand, it is demonstrated that patients with other mutation of LRP5 gene have high bone mass [8]. In this mutation,

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the mutated LRP5 receptor has a low binding affinity to Dkk-1 and causes decrease in inhibitory function of Dkk-1 against Wnt signaling. Thus, the Wnt signal is now recognized as a novel regulator of bone formation and an important molecular target for the treatment of osteoporosis [9,10].

Glucocorticoid-induced osteoporosis is one of the serious problems during glucocorticoid therapy [11,12]. The major cause of glucocorticoid-induced osteoporosis is considered to be impairment of bone formation [11,12]. Glucocorticoid in an excess dose has inhibitory actions on osteoblastic replication, maturation, and differentiation. Glucocorticoid suppresses the expression of *Runx2/Cbfa1*, a critical factor for osteoblastogenesis, and reduces the synthesis of osteocalcin, type I collagen, and insulin-like growth factor-I (IGF-I) in osteoblasts [11,12]. It also promotes the apoptosis of osteoblasts and osteocytes [13]. However, detailed mechanism underlying glucocorticoid-induced osteoporosis remains to be fully elucidated.

In the present study, we hypothesized that glucocorticoid would affect the Wnt signal of bone formation in osteoblasts, and examined the effect of glucocorticoid on the expression of Wnt signal-related molecules in primary cultured human osteoblasts. We found that dexamethasone induces the expression of Dkk-1, an antagonist of Wnt, through the activation of trascription via glucocorticoid responsive element (GRE) of the Dkk-1 gene promoter.

## Materials and methods

*Materials.* Eagle's α MEM, penicillin, and streptomycin were obtained from Invitrogen (Carlsbad, CA). Fetal calf serum (FCS) was purchased from Sanko Junyaku (Tokyo, Japan). Dexamethasone, 17β-estradiol, dihydrotestosterone, and 1,25-dihydroxyvitamin D3 were purchased from Sigma (St. Louis, MI). All other reagents were of analytical grade.

*Cell culture.* Human osteoblasts were prepared from the bone fragments of femur neck as described previously [14]. The cells were grown in Eagle's α MEM with 10% FCS, 100 mU/ml penicillin, and 100 mU/ml streptomycin. Cells at ~80% confluence in 100-mm culture dishes (Falcon, Lincoln Park, NJ) were made quiescent through incubation with serum-free medium for 1 day before experiments.

*Quantification of RNA.* Total RNA was isolated from cultured cells using a RNeasy RNA Extraction kit (Qiagen, Germany). Reverse transcriptase-polymerase chain reaction (RT-PCR) and Northern blot analysis were performed as described previously [14,15]. The primer sets for DKK-1 (396 bp) were 5′-TGATGAGTACTGCGCTAGTC-3′ (sense) and 5′-CTCCTATGCTTGGTACACAC-3′ (antisense), the primer sets for LRP5 (377 bp) were 5′-CCGTCATTGGCATCA TCCTC-3′ (sense) and 5′-GTCCATGTTGTACAGGGAGG-3′ (antisense), the primer sets for Kremen1 (354 bp) were 5′-GTTTGCTGGG ATGGAGTCAG-3′ (sense) and 5′-GTGTAGCCATCCAGAAGC TC-3′ (antisense), and the primer sets for GAPDH (321 bp) were 5′-GGGCTCTCCAGAACATCATC-3′ (sense) and 5′-CAAAGTGGTC GTTGAGGGCA-3′ (antisense). The PCR products were subcloned into the pGEM-T Easy vector (Promega, Madison, WI), sequenced, and then used as cDNA probes for Northern blot analysis.

Determination of the initiation site of transcription for the Dkk-1 gene was performed by a RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE) kit (Ambion, Austin, TX) according to the manufacturer's instruction. The outer and inner antisense primers used for 5′ RLM-RACE for Dkk-1 were 5′-CTGCAGGCGAGACAGA TTTG-3′ and 5′-GGCTGGTAGTTGTCAATGGT-3′, respectively.

*Construction of human Dkk-1 promoter/luciferase chimeric plasmids.* Human gemonic DNA was purified from a Japanese man with a QIAamp DNA Blood Kit (Qiagen). The approximately 0.8 kb upstream region of the human Dkk-1 promoter was amplified by PCR using KOD-plus DNA polymerase (Toyobo, Tokyo, Japan), subcloned into the pCR-Blunt II-TOPO vector (Invitrogen), and sequenced to confirm the validity of the PCR product. The primer sets were 5′-CTCACGCGTCTGCCTAATCA-3′ (sense) and 5′- AAGCTTTCAG AAGGACTCAAGAGGGA-3′ (antisense, *Hin*dIII-linker added). After digestion with *Mlu*I and *Hin*dIII, the fragment was subcloned into the *Mlu*I/*Hin*dIII site of a promoterless luciferase expression vector, pGL3-Basic vector (Promega) and designated as pGL3-Dkk-1(−837/+151). Serial 5′-deletion constructs of the Dkk-1 promoter were generated by PCR, using pGL3-Dkk-1 (−837/+151) as template. To generate the mutant, PCR-mediated site-directed mutagenesis was performed by a QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). All the deletion and mutation constructs were confirmed by DNA sequencing.

*Transient transfection and reporter assay.* Human osteoblasts were transiently transfected by means of calcium phosphate precipitation [16]. Briefly, cells ($5 \times 10^4$ cells/well) were seeded in a 12-well plate (Falcon) prior to transfection: each well received 5 μg of the Dkk-1 promoter-reporter firefly luciferase plasmid (pGL3) and 100 ng pRL-CMV (a *Renilla* luciferase vector, Promega) as an internal control. Two hours after transfection, the cells were incubated in Eagle's α MEM with 10% dextran/charcoal-treated FCS in the presence or absence of $10^{-7}$ M dexamethasone. Firefly and *Renilla* luciferase activities were measured at 36 h after transfection with a dual luciferase assay kit (Promega), and the values were adjusted for the activity of the internal control (*Renilla* luciferase activity).

*Statistical analysis.* Data are expressed as means ± SD. Statistical analyses were performed with ANOVA followed by Fisher's protected least significant difference test. Significance was accepted at $P < 0.05$.

## Results

We first screened the mRNA expression for the Dkk family (Dkk-1, -2, -3, and -4) by RT-PCR, and found that only Dkk-1 mRNA is expressed in primary cultured human osteoblasts (data not shown). We then examined the expression level of Dkk-1 mRNA by Northern blot analysis (Fig. 1). Although quiescent human osteoblasts expressed a low level of Dkk-1 mRNA transcript (1.8 kb), dexamethasone ($10^{-7}$ M) significantly induced the expression of Dkk-1 mRNA (more than 10-fold) compared to that in unstimulated condition (Fig. 1A). This effect was observed at 6 h and reached maximum at 24 h (Fig. 1B). This increase was in a dose-dependent manner ($10^{-9}$–$10^{-7}$ M of dexamethasone) (Fig. 1A). We also examined the effect of other steroid hormones on the expression of Dkk-1 mRNA. Addition of $10^{-7}$ M of 17β-estradiol (E2), dihydrotestosterone (DHT) or 1,25-dihydroxyvitamin D3 (VD3) did not affect the expression level of Dkk-1 mRNA (Fig. 2). These results

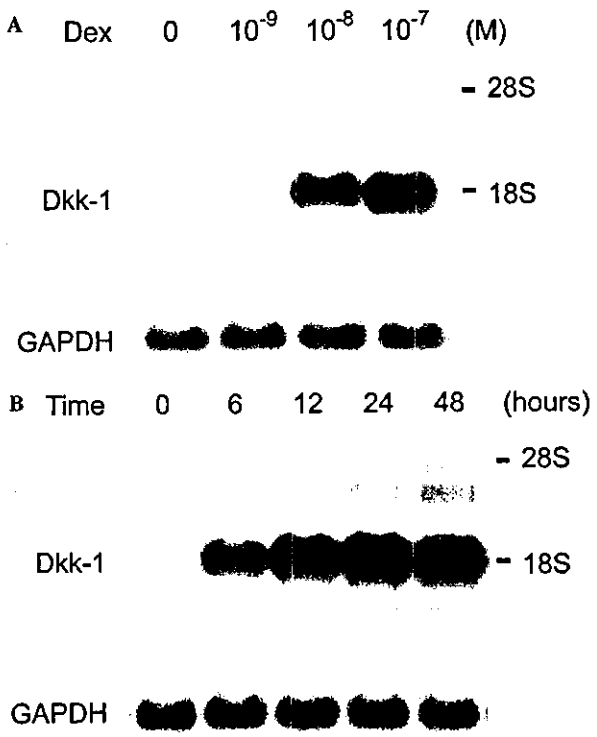


Fig. 1. Effect of dexamethasone on the expression level of Dkk-1 mRNA in primary cultured human osteoblasts. (A) Human osteoblasts were incubated for 24 h with vehicle (ethanol) or dexamethasone (Dex, $10^{-9}$–$10^{-7}$ M). (B) Human osteoblasts were incubated for 6–48 h with dexamethasone ($10^{-7}$ M). Extracted total RNA (2 μg in each lane) was subjected to Northern blot analysis. A representative autoradiograph of three independent experiments are shown.

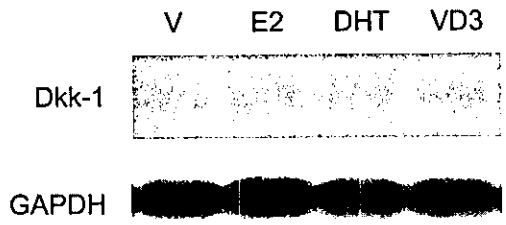


Fig. 2. Effect of various steroid hormones on the expression level of Dkk-1 mRNA in primary cultured human osteoblasts. Human osteoblasts were incubated for 24 h with vehicle (V, ethanol), 17β-estradiol (E2, $10^{-7}$ M), dihydrotestosterone (DHT, $10^{-7}$ M), or 1,25-dihydroxyvitamin D3 (VD3, $10^{-7}$ M). Extracted total RNA (2 μg in each lane) was subjected to Northern blot analysis. A representative autoradiograph of three independent experiments is shown.

suggest that glucocorticoid specifically induces the expression of Dkk-1 mRNA in primary cultured human osteoblasts.

We then examined whether dexamethasone affects the expression of other Wnt signal-related molecules in cultured human osteoblasts. Cultured human osteoblasts expressed the mRNA transcript for Kremen1, a receptor for Dkk [17], and that for LRP5. As shown in Fig. 3, dexamethasone ($10^{-7}$ M) did not alter the expression level of the mRNA for Kremen1. On the other hand, the expression of LRP5 mRNA slightly decreased by the treatment with dexamethasone ($10^{-7}$ M).

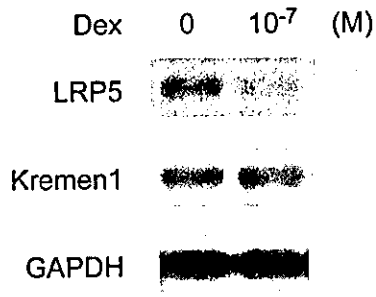


Fig. 3. Effect of dexamethasone on the expression levels of LRP5 and Kremen1 mRNA in primary cultured human osteoblasts. Human osteoblasts were incubated for 24 h with vehicle (ethanol) or dexamethasone (Dex, $10^{-7}$ M). Extracted total RNA (2 μg in each lane) was subjected to Northern blot analysis. A representative autoradiograph of three independent experiments is shown.

To clarify the mechanism by which dexamethasone up-regulates the expression of the Dkk-1 mRNA, we investigated the effect of dexamethasone on the promoter activity of the human Dkk-1 gene in cultured human osteoblasts. At first, we determined the transcription initiation site for the Dkk-1 gene in human osteoblasts by RLM-RACE. One major product was observed by PCR, and the sequence analysis of this product revealed that the transcription initiation site is 'A' at −152 bp relative to the translation start site. We then examined the 5′-promoter region on the transcriptional activity. When the reporter plasmid containing of the 0.8 kb upstream region was transfected into human osteoblasts, the basic promoter activity was 8–10 times higher than that of promoterless control plasmid. The addition of dexamethasone ($10^{-7}$ M) increased 6–8 times the luciferase activity of the 0.8 kb construct (Fig. 4). The magnitude of the enhancement of the dexamethasone-induced transcription was comparable to that of the Dkk-1 mRNA induction by dexamethasone (Fig. 1A). Deletion of the 5′-promoter region from −837 to −540 bp, which contains one of three putative GRE-like sequences located within the 0.8 kb upstream region, abolished the effect of dexamethasone. No significant change of the dexamethasone effect on the promoter activity was observed by the deletion from −540 to −409 bp and that from −409 to −314 bp, each of which contains one putative GRE (Fig. 4). Mutation of the GRE-like sequence (−788 to −774 bp) in the promoter region from −837 to −540 bp also abrogated the induction effect of dexamethasone. These results suggest that the GRE located from −788 to −774 bp functions in the induction of Dkk-1 in response to glucocorticoid in human osteoblasts.