Implications of arginine deficiency for growth and organ maturation. Studies on hair, muscle, brain and lymphoid organ maturation

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Chapter III

Overexpression of arginase I in enterocytes of transgenic mice elicits a selective arginine deficiency and affects skin, muscle and lymphoid development

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Abbreviations used in this paper:

CPS [EC 6.3.4.16] : carbamoylphosphate synthetase
OTC [EC 2.1.3.3] : ornithine transcarbamoylase
ASS [EC 6.3.4.5] : argininosuccinate synthetase
ASL [EC 4.3.2.1] : argininosuccinate lyase
A-I [EC 3.5.3.1] : hepatic arginase
A-II [EC 3.5.3.1] : non-hepatic arginase
OAT [EC 2.6.1.13] : ornithine aminotransferase
SI [EC 3.2.1.48-10] : sucrase-isomaltase
NOS : nitric oxide synthase
FABPi : intestinal fatty acid-binding protein
GALT : gut-associated lymphoid tissue
ND : neonatal day, days after birth
PP : Peyer’s patch
Summary

Background. Arginine is required for the detoxification of ammonia and the synthesis of proteins, nitric oxide, agmatine, creatine and polyamines, and may promote lymphocyte function. In suckling mammals, arginine is synthesized in the enterocytes of the small intestine, but this capacity is lost after weaning. Objective. We investigated the significance of intestinal arginine production for neonatal development in a murine model of chronic arginine deficiency. Design. Two lines of transgenic mice which express different levels of arginase I in their enterocytes, were analyzed. Results. Both lines suffer from a selective, but quantitatively different reduction in circulating arginine concentration. The degree of arginine deficiency correlated with the degree of retardation of hair- and muscle growth, and the development of the lymphoid tissue, in particular Peyer’s patches. Expression of arginase in all enterocytes was necessary to elicit this phenotype. Phenotypic abnormalities were reversed by daily injections of arginine, but not creatine. The expression of the very arginine-rich skin protein trichohyalin is not affected in transgenic mice. Finally, NOS-deficient mice did not show any of the features of arginine-deficiency.

Conclusions: 1) Enterocytes are important for maintaining arginine homeostasis. 2) Graded arginine deficiency causes graded impairment of skin, muscle and lymphoid development. 3) The effects of arginine deficiency are not mediated by impaired synthesis of protein or creatine.
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Introduction

Arginine is a precursor for the synthesis of proteins, NO, agmatine, creatine and polyamines, and an intermediate in the detoxification of ammonia. Of these, protein, creatine and urea synthesis are quantitatively most important. Endogenous arginine biosynthesis in adult mammals occurs mainly in the proximal convoluted tubules of the kidney (1) from citrulline (2) that, in turn, is formed in the enterocytes of the small intestine from glutamine and proline (3-5). The endogenous biosynthesis of arginine is low in strict carnivores such as cats and ferrets, intermediate in humans, and relatively high in rapidly and continuously growing animals such as rats and pigs (6). In adult humans, the endogenous biosynthetic capacity for arginine amounts to approx. 20% of daily requirement (7), which barely meets daily demand under steady state conditions. A dietary source of arginine may therefore become necessary when demand increases under anabolic or catabolic conditions (7). For this reason, arginine is coined as a conditionally essential amino acid.

In rapidly growing suckling rats, the dietary supply of arginine via the milk does not suffice to meet the requirement for arginine accumulation in proteins (8, 9), implying that endogenous arginine biosynthesis must be important during this period. Evidence is accumulating that the intestine rather than the kidney plays a major role in arginine biosynthesis in the suckling period. In concert, the enterocytes of the small intestine express the enzymes required for arginine production from glutamine and proline (5, 10-14) and do not express arginase (15). The intestine not only appears to play a crucial role in arginine biosynthesis in rapidly growing neonates like rodents and pigs, but also in neonatal humans, as destruction of the enterocytes in necrotizing enterocolitis also results in a selective decrease in circulating arginine (16). After weaning, argininosuccinate synthetase and argininosuccinate lyase, the enzymes that synthesize arginine from citrulline, disappear from the enterocytes, concurrent with the appearance of endogenous arginase (15), so that only the capacity to synthesize citrulline remains.

Arginine deficiency is known to cause a life-threatening hyperammonemia in preterm infants (17). Furthermore, arginine deficiency in growing animals is usually associated with growth retardation (12, 13, 15). However, it is not known if arginine
deficiency also affects developmental processes other than growth, and if so, what the quantitative relation between arginine deficiency and these sequellae is. Furthermore, the regulatory mechanisms underlying the development of these sequellae in growing mammals have to be solved to gain a better understanding of the role of arginine metabolism in normal growth, vessel-wall biology (18, 19) and immunological responsiveness (20, 21). We therefore developed a transgenic mouse model, which suffers from a selective decrease in circulating and tissue arginine as a result of overexpression of hepatic arginase I (A-I) in the enterocytes of the small intestine. The transgenic model highlights the importance of the small intestine for arginine metabolism in the suckling period. It further shows that a graded deficiency of arginine results in a graded deficiency in the growth and development of hair, muscle and immune system, but that these consequences do not arise from a deficient synthesis of protein, creatine and probably nitric oxide, or from an excess of ammonia.
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Animals

Mice. Mice were kept under environmentally controlled conditions (light on from 8:00 a.m. to 8:00 p.m.; water and rodent chow ad lib; 20-22°C, 55% humidity). Litters discovered in the morning were assigned ND0. Pups were weaned at 3 weeks of age. For generation of transgenics, mice of the FVB-strain were used. Spf-ash and NOS1-, 2- and 3-deficient mice were of C57/Bl6 background and purchased from Jackson Laboratories, Bar Harbor, Maine. Animal experiments were performed in accordance with the guidelines of the local Animal Research Committee.

Supplementation and weight curves. Nest-size was adjusted to 7 pups. Five mmol/kg of 150 mM arginine-HCl or creatine-monohydrate was injected subcutaneously on the back twice daily (9:00 a.m. and 4:00 p.m.). Control animals were injected with the same volume of 0.9% NaCl. Animals were weighed daily at 3:00 p.m. Each weight curve is based on repeated measurements of 10-16 animals. Male and female weight curves did not differ significantly.

Methods

Targeting arginase I expression to the enterocytes. The 4.3 kb construct used for generation of the transgenic animals (Fig. 1) is a chimera of the -1,178 to +28 bp fragment of the rat FABPI promoter/enhancer element (22), 520 bp rat A-I cDNA, containing exons 1-5, and 2,400 bp of genomic rat A-I DNA, containing exons 5 to 8 of rat hepatic arginase (23). PCR of tail-tip DNA was carried out at 56°C using 5'-AAATGCCTACATGCTGTAGTCGG-3', complementary to nucleotide -218 to -196 of the FABPI promoter/enhancer DNA as upstream primer, and 5'-CCAATTGCCATACTGTGGTCTCC-3', complementary to nucleotide +320 to +342 of the arginase I cDNA as downstream primer. Transgene copy number was determined by Southern-blotting and was 5-6 in both lines studied. Putative homozygous mice were crossed with wild-type mice, and a sample of 15 pups was analyzed by PCR screening to confirm the genotype. Homozygous mice of line F/A-1 and hemizygous mice of line F/A-2 were used for breeding. Homozygous F/A-2 mice were identified by phenotype and weight (Fig. 2).
arginine in growth and development

Fig. 1. Chimeric FABPi-arginase DNA construct used to generate F/A transgenic mice. Exons are indicated by black boxes. The 4.3 kb construct consists of a chimera of the -1178 to +28 bp EcoRI-BamHI rat intestinal FABPi promoter/enhancer fragment (61), the 520 bp PstI-BsrGI rat A-I cDNA fragment containing exons 1-5, and the 2400 bp BsrGI-HindIII fragment of genomic rat A-DNA, containing exons 6-8 (23).

**Determination of amino acid, creatine and ammonia concentration.** Pups were separated from their mother and kept at 37°C for 1 hour prior to sacrifice. After decapitation, blood was collected into heparin-containing tubes and centrifuged at 2,000 x g for 5 min at 4°C. Fifty μL of plasma was added to 4 mg of lyophilized sulphosalicylic-acid, centrifuged, and stored at -70°C. Tissue samples were collected, flushed in ice-cold PBS, rapidly frozen in liquid N₂ and stored at -70°C. Approximately 80 mg of tissue was added to 400 μL of a 5 % sulphosalicylic acid solution, containing 300 mg glass beads with a diameter of 1 mm. The tissue was homogenized for 30 sec in a Mini Bead-Beater (BioSpec Products, Bartesville, OK) and centrifuged. Jejunum and plasma amino acids were determined by fully automated HPLC as described (24). Norvaline was used as an internal standard. Cadaverine was determined to assess residual bacterial content in the intestinal lumen. For analysis of guanidino compounds, plasma was deproteinized with an equal volume of 20 % trichloroacetic acid. Guanidino compounds were separated on a cation-exchange column and detected as described (25). Urea nitrogen was determined with diacetylmonoxime, as described (26). Blood ammonia concentration was determined using the ammonia test kit (Menarini Diagnostics, Florence, Italy), according to the manufacturer’s instructions.
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Histology. For in situ hybridization, tissues were fixed in 4% formaldehyde in PBS for 4 hours at 4°C, and overnight at room temperature. In situ hybridizations were carried out as described (27). The A-I probe was the 768 bp PstI-Stul fragment of clone pARGr-2 (28). For immunohistochemistry, tissues were fixed in methanol/acetone/water (2:2:1; v/v) for 4 hours at 4°C and overnight at room temperature, embedded in paraplast and sectioned at 7µm. After deparaffination, endogenous peroxidase activity was eliminated by treatment with 3% H2O2 in 50% methanol. Non-specific binding was blocked by incubation for 30 min in TENG-T (10mM Tris, 5 mM EDTA, 150 mM NaCl, 0.25% gelatin, 0.05% Tween-20, pH 8.0). Serial sections of intestines were incubated overnight with an appropriate dilution of a rabbit antiserum against ASS (29, 30), A-I (31), CPS (32) and SI (33). Rabbit anti-rat A-II antiserum was raised against amino acids 337-350 of rat A-II (34), coupled to keyhole limpet hemocyanin. The indirect unconjugated peroxidase-anti-peroxidase technique (35) was used to visualize binding of the primary antibodies. Sections of skin were stained with a polyclonal antibody against trichohyalin (36). For whole-mount immunostaining, small intestines of ND6 mice were fixed for 16 hrs in methanol/DMSO (4:1) at 4°C with gentle agitation and incubated with a monoclonal FITC-conjugated antibody against murine VCAM-1 (1 µg/ml in PBS; PharMingen, San Diego, CA). After washing, tissues were incubated for 2 hrs at 37°C with HRP-conjugated anti-FITC antibodies (POD converter kit, Boehringer Mannheim) and stained with diaminobenzidine and H2O2 in the presence of NiCl2.

Western blotting. Jejunal tissue and shaven skin was homogenized in 20 mM phenylmethylsulphonyl fluoride, 1 mM EDTA and 1 mM DTT. Twenty µg of protein (BCA assay, Pierce, Rockstone, IL) was separated on 10% SDS polyacrylamide gels and blotted onto polyvinylidene difluoride membranes (Millipore Corporation, Bedford, MA). Membranes were blocked by overnight incubation in 10% non-fat milk in PBS and subsequently incubated with primary antibodies. Goat anti-rabbit-IgG conjugated to alkaline phosphatase was used as secondary antibody.
Quantification of serum immunoglobulin concentration. Serum was collected from 8 and 18 day-old wild-type and homozygous F/A-2 littermates by decapitation. Total IgG concentration was quantified using an ELISA-based assay according to the manufacturer’s instructions (Southern Biotechnology Associates, Birmingham, AL).

Arginase activity assay. Intestinal tissue was homogenized in 40 volumes of 30 mM sucrose, 5 mM MnCl₂, 30 mM imidazole buffer (pH 7.5), and 0.05 % Triton X-100. This homogenate was sonicated on ice for 2 min, activated at 55 °C for 5 min and centrifuged. The assay mixture (200 µL) contained enzyme extract, 25 mM (guanidino-¹⁴C)-L-arginine (pH 9.5, 15 mCi.mol⁻¹; Dupont NEN, Boston, MA), and 75 mM glycine (pH 9.5). After 10 min at 37 °C, the reaction was stopped by loading the assay mixture on a cation-exchange column (BioRad, Richmond, CA). Urea was eluted with 1 ml of Tris-EDTA buffer, pH 8.5, and counted. Protein content was determined with the BCA assay.

Statistics. Weight data were fitted to a logistic growth curve (weight = weightₘₐₓ / (1 + e⁻ᵇᵗ₋₁₀)). Fitting the curve in two stanzas (preweaning and postweaning) resulted in a significantly better fit (F-test on reduction of residual variation). To compare the resulting bi-phasic weight curves between genotypes and/or treatments a 99 % confidence interval was constructed both at 3 weeks and at 7 weeks. Biochemical data on arginase, amino acids, immunoglobulins and guanidino compounds were tested with a repeated measure analysis of variance (ANOVA) per compound group. Because of significant interactions between compound, genotype and age, a two-way ANOVA (arginase, amino acids, and immunoglobulins; factors age and genotype) or a one-way ANOVA (guanidino compounds; factor genotype) was carried out per compound. In case of a significant effect of genotype or genotype-age interaction in these ANOVAs, Dunnett’s multiple comparison test between genotypes was applied. Results were considered significantly different at p<0.01. Counts on Peyer's patches were tested with a Poisson distribution.
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Results

*Intestinal arginase expression is associated with retarded body growth and retarded fur development*

Six founder-mice were bred and analyzed. Two lines, designated F/A-1 and F/A-2, were clearly distinguishable from age-matched wild-type animals in that the appearance of a normal fur coat was delayed until after weaning in homozygous F/A-1 and all F/A-2 mice (Fig. 2A-D). The animals are no longer distinguishable from wild-type animals by 6-7 weeks. Lines F/A -3 to -6 were indistinguishable from wild types. Histological analysis of homozygous F/A-2 skin at weaning revealed a severe distortion of hair follicles (Fig. 3A,D). Many hair bulbs lie too high in the subcutaneous tissue, contain keratohyalin grains and do not develop normally. Prominent keratin plugs clog the hair follicles. In general, the appearance is that of a delayed maturation of hair. However, the onset of sebaceous gland development appears normal. The presence of hyperkeratosis and a prominent stratum granulosum in the epidermis also points to delayed maturation. The hair follicle protein trichohyalin is extremely rich in arginine residues (36) and, hence, a potential target of arginine deficiency. However, immunohistochemical staining of skin sections (Fig. 3B and E) and Western-bLOTS of skin homogenates (Fig. 3C and F) did not reveal any differences in trichohyalin content, though we did observe absence of trichohyalin from the epidermis.

Mice show a biphasic growth curve in the first 8 weeks of life, with most rapid growth occurring immediately after birth and after weaning (Fig. 2E). The growth rate of homozygous F/A-1 and hemizygous F/A-2 mice is normal, but homozygous F/A-2 animals exhibit a pronounced retardation in body growth before weaning, which becomes discernible at 5 days after birth and which amounts to a 2-fold reduction in body weight at weaning. Since muscle is an important component of body weight, we analyzed the gastrocnemius muscle of 3-weeks-old homozygous F/A-2 mice histologically (Fig. 3G-J). The diameter of the myofibers is approx. 3-fold smaller than that of wild types, with many nuclei located in the periphery of the fibers.
Fig. 2. Appearance and body weight of F/A-1 and F/A-2 transgenic mice. Homozygous (+/+ ) and hemizygous (+/wt) F/A-1 mice are shown in panel A, and homozygous and hemizygous F/A-2, and WT (wt/wt) mice in panel B, all at ND20. Note the difference in fur development and body size, in particular the similarity of F/A-1"""" and F/A-2"""" mice, and of F/A-1"""" and wild-type mice. Panel C: injection of arginine (right mouse), but not PBS (left mouse) restores fur development in F/A-1"""" mice. Panel D: injection of arginine causes recovery of F/A-2"""" mice (left animal), but only partial recovery of F/A-2"""" mice (right animal). Panel E: weight gain of F/A transgenics: WT (○), F/A-1"""" (□), F/A-2"""" (□), F/A-2"""" (△).
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2''' mice have a significantly (p<0.01) lower body weight at weaning, but fully recover as adults. Panel F: weights of PBS- (▲) and arginine-injected (▼) F/A-2''' mice, compared to wild-type animals (◇). At ND21, arginine-injected F/A-2''' mice showed significantly increased body weight, compared to PBS-injected F/A-2''', but did not reach wild-type weight (for both: p<0.01). Panel G: Serum total IgG concentration in wild-type and F/A-2''' mice at ND8 and ND18. Black bars, wild type; gray bars, F/A-2'''. Serum total immunoglobulin concentration is similar in both genotypes. For each measurement, serum of three mice was pooled. Values are ± SEM and based on three independent analyses. Panel H: weight curve of OTC-deficient spf-ash mice (◆) and wild-type C57Bl6 mice (◇). At ND21, body weight of spf-ash mutant mice is significantly (p<0.01) lower than that of wild-type mice. Error bars depict a 99 % confidence interval of fitted growth curves.

Since no dystrophic features were observed, the picture is suggestive for active myogenesis.

Suckling homozygous F/A-2 mice appear to have a normal feeding behaviour and intestinal function. The absence of malnutrition was deduced from the presence of normally filled stomachs and a normal serum concentration of immunoglobulins (Fig. 2G), which are of maternal origin and have to be transported across the intestinal epithelium (37). Furthermore, the transgenic mice are not feeble but, instead, hyperactive (see Chapter IV).

The fur of the F/A transgenics resembles that of the OTC-deficient sparse-fur-abnormal-skin-and-hair (spf-ash) mutant mice (38). The growth rate of suckling spf-ash mice is also retarded significantly, but to a lesser extent than in F/A-2 mice (Fig. 2H).

**Intestinal arginase expression selectively reduces arginine concentration**

We confirmed that CPS and OTC are expressed in all enterocytes of suckling mice, and that ASS and ASL are only expressed in enterocytes on the distal half of the villi, as we have previously reported for the suckling rat intestine (15). As in suckling rats, endogenous arginase activity in the intestine does not become detectable until the third postnatal week in mice (Fig. 4A). Arginase activity in homozygous F/A-1 mice already amounts to 0.2 U/mg protein shortly after birth and rises to 0.7-0.8 U/mg protein in the second postnatal week.
Fig. 3. Skin and muscle development in wild-type and F/A-2+/- mice. Panels A and D show haematoxylin and azophloxin-stained sections, and panels B and E sections of dorsal skin stained for the presence of trichohyalin in wild-type (A, B) and F/A-2+/- (D, E) mice. Note the disturbed appearance of hair follicles in transgenic mice, but normal staining pattern of trichohyalin in the inner root sheath in transgenic animals. Panels C and F: Western blot of skin extract stained for the presence of trichohyalin. Note similar concentration in both extracts. Panels G-J show haematoxylin and azophloxin-stained sections of gastrocnemius muscle of wild-type (G, H; transverse sections) and F/A-2+/- mice (I, J; longitudinal sections) at ND20. Note the regular myofiber pattern, with peripherally located nuclei, in both genotypes, but also the 3-fold decrease in fiber diameter and the pronounced increase in number of peripheral nuclei in F/A-2+/- mice. Bar: 100 μm.

Arginase activity in line F/A-2 follows a similar pattern, but at a 2-3-fold higher concentration. The accelerated rise in enzyme activity in F/A-1 and -2 is at least partly due to the rise in endogenous arginase. Apparently, the expression of A-I does not interfere with the expression of endogenous arginase. At day 20, endogenous arginase activity is highest in duodenum, whereas FABPi-driven A-I activity is highest in the jejunum and decreases towards the stomach proximally and the colon distally (Fig.
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4B). Hemizygous mice express arginase at an intermediate level between wild-type and homozygous mice (Table 1).

![Graph showing arginase activity over age (days) for different genotypes.]

**Fig. 4. Appearance and distribution of arginase activity in the small intestine.** Panel A: arginase activity in jejunal homogenates of wild-type and transgenic animals: F/A-2""“ (▲), F/A-1"""" (●), wild-type (○). Panel B: distribution of arginase activity along the proximal-distal axis of the intestine in wild-type (white bars), F/A-1"""" (gray bars), and F/A-2"""" (black bars) mice at ND20. Note that the peak of FABP-driven A-I activity is found in the jejunum, whereas endogenous arginase expression is highest in the duodenum. Panels C-F show distribution of A-I mRNA and panels G-J of A-I protein in jejunal enterocytes of wild-type animals (C, G), F/A-1"""" (D, H) and F/A-2"""" (E, I), and F/A-3"""" (F, J) at ND8. Note that lines F/A-1 and -2 express A-I in all enterocytes, whereas F/A-3 expresses A-I in approximately 5% of the enterocytes. Bar: 75 μm.

**Table 1. Intestinal arginase activity in F/A transgenic mice.**

<table>
<thead>
<tr>
<th>line</th>
<th>A-I activity (mU/g prot.)</th>
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<tbody>
<tr>
<td>WT</td>
<td>7 ± 3</td>
</tr>
<tr>
<td>F/A-1</td>
<td>208 ± 3</td>
</tr>
<tr>
<td>F/A-2</td>
<td>450 ± 6</td>
</tr>
<tr>
<td>F/A-3</td>
<td>289 ± 31</td>
</tr>
<tr>
<td>F/A-4</td>
<td>169 ± 26</td>
</tr>
<tr>
<td>F/A-5</td>
<td>84 ± 17</td>
</tr>
<tr>
<td>F/A-6</td>
<td>221 ± 47</td>
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</tbody>
</table>

Arginase activity (nmol/min/mg prot) was determined in hemizygous animals of the 6 available lines at ND12. Data represent the mean ± SEM of 6 mice. *: indicates statistical difference from wild type at p < 0.05.
The sparse-fur phenotype of F/A-1 and F/A-2 mice is associated with A-I expression in all enterocytes, except those in the crypts (Fig. 4D,E,H,I). Hence, transgenic A-I colocalizes with ASS and ASL. Transgenic A-I protein or mRNA is not expressed in other tissues, such as the kidney, in these lines. Table 1 shows the intestinal arginase activities in hemizygous F/A-3 to -6 mice, which do not have a phenotype. Arginase activity in lines F/A-3 and -6 exceeds that present in F/A-1 mice but, in contrast to line F/A-1, A-I is expressed in only 5% of the enterocytes (Fig. 4F, J). These data show that, in addition to a high activity of A-I, a homogenous expression of A-I is necessary for the F/A phenotype to develop.

In suckling wild-type mice, the concentration of arginine is 200-250 μM in plasma and 350-400 μmol/kg wet weight in jejunal tissues (Fig. 5A). In the homozygous suckling F/A-1 and -2 mice, plasma and jejunal arginine concentrations are reduced to, respectively, 90-110 and approximately 75 μM, and 110-140 and approximately 80 μmol/kg. Arginine concentration in F/A-1 mice returns to wild-type shortly after weaning. In homozygous F/A-2 mice arginine concentration also rises significantly after weaning in females (P < 0.01), but it remains depressed in males. Fig. 5B shows the relation between intestinal arginase activity and the corresponding arginine concentration in plasma or jejunum. A “sparse-fur” phenotype is only seen if intestinal arginase activity exceeds 0.6 U/mg protein, or if the circulating arginine concentration drops below 120 μM. Furthermore, small additional decreases in arginine concentration apparently suffice to cause a pronounced aggravation of the phenotype.

Arginine is the only amino acid of which the decrease in jejunum and plasma concentration corresponds with increasing transgenic arginase concentration in the enterocytes (Fig. 5B). Overexpression of arginase in the enterocytes is accompanied by a selective increase in the arginase product ornithine in jejunum of almost two-fold (p<0.01). Except a 45% and 20% increase in plasma glycine and tryptophan respectively, the plasma concentration of all other amino acids is similar in F/A and wild-type mice prior to weaning (not shown). The accumulation of ornithine is not associated with an increased tissue concentration of the polyamines spermine, spermidine or putrescine (not shown).
Fig. 5. Arginine concentration in plasma and jejunum of wild-type and F/A-transgenic mice. Panel A shows arginine concentration in plasma and jejunum of wild-type ( ), F/A-1+/wt ( ), F/A-1+ ( ), F/A-2−+/wt ( ) and F/A-2−/+ ( ) mice as a function of age. Note that arginine concentration in transgenic animals is low during the suckling period, but recovers after weaning, except in male F/A-2−/+ mice. Panel B: plasma and jejunal arginine concentration as a function of jejunal arginase activity in wild-type, F/A-1−/−, F/A-1−/−, F/A-2−/−, and F/A-2−/+ mice. On the Y-axis, plasma and jejunal arginine concentration are given in μM and μmol/kg wet weight, respectively.

The plasma concentrations of urea, the other arginase product, and of ammonia are not elevated (Table 2). Table 2 also shows that the concentrations of two other products of arginine metabolism, creatine and creatinine, known to be sensitive to decreases in plasma arginine (39, 40), are almost halved in both F/A-1 and -2 mice.
The decreased creatine biosynthesis may well explain the increased concentration of its precursor glycine.

<table>
<thead>
<tr>
<th></th>
<th>Wild-type</th>
<th>F/A-1(^{+/+})</th>
<th>F/A-2(^{+/+})</th>
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<tbody>
<tr>
<td>Arginine</td>
<td>295±27</td>
<td>59±5(*)</td>
<td>63±8(*)</td>
</tr>
<tr>
<td>Ammonia</td>
<td>39±11</td>
<td>40±11</td>
<td>44±14</td>
</tr>
<tr>
<td>Urea</td>
<td>8.1±0.1</td>
<td>8±1</td>
<td>10±2</td>
</tr>
<tr>
<td>CT</td>
<td>406±33</td>
<td>218±2(*)</td>
<td>226±70(*)</td>
</tr>
<tr>
<td>CTN</td>
<td>18±2</td>
<td>11±1(*)</td>
<td>9±1(*)</td>
</tr>
</tbody>
</table>

Table 2. Products of arginine metabolism in F/A transgenic mice. Concentrations were measured in serum of 10 days old animals (n=3 pools of 3 animals each for each genotype). \(*\): indicates statistical difference from wild type at p < 0.05

**Effect of arginine supplementation**

To demonstrate that the F/A phenotype results from arginine deficiency, mice of both F/A-1 and -2 lines were supplemented twice daily with arginine from neonatal day 3 onwards. For development and body growth of homozygous F/A-1 animals and hemizygous F/A-2 mice completely normalized and that of homozygous F/A-2 mice partly (Figs. 2C,D,F). Circulating plasma arginine concentrations in ND10 mice, 6 hours after the last injection, were 560, 120 and 90 μM in wild-type, hemizygous and homozygous F/A-2 animals, respectively (n=3 each). These data show that the transgenic A-I activity in the enterocyte also functions as an effective drain for circulating arginine. As creatine concentration is reduced twofold, we also tested supplementation with creatine, but this treatment was without effect.

**Maturation of intestinal mucosa in transgenics**

The normal absorption of maternal immunoglobulins (Fig. 2G) suggests that the gut of suckling F/A-2 mice functions properly. We assessed periweaning maturation of the enterocytes of the small intestine by monitoring the change in expression of A-II and SI, which normally starts at ND16 and reaches mature levels at ND21, and of ASS, which follows a reciprocal course (15). A-II and SI follow their normal developmental course in homozygous F/A-2 (Fig. 6A) and F/A-1 mice (not shown). However, ASS expression, which has become undetectable in wild types at ND20, is still detectable at this age in F/A-2 mice, but not thereafter (Fig. 6A). This temporary
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Persistence of ASS expression may well reflect a response to the limited bioavailability of arginine, since a similar feedback mechanism was recently reported for rat kidney (41).

We also assessed the pattern of expression of SI and ASS along the villus-crypt axis (Fig. 6B,C). Periweaning maturation of the small intestine starts in both wild type and homozygous F/A-2 mice at ND16 with the appearance of SI at the base of the villi and the disappearance of ASS from this location. The spread of SI expression to the apex of the villi remains tightly associated with the disappearance of ASS, but follows a slightly slower course in the F/A-2 mice compared to the wild-type animals. ASS was only found to be expressed in immature enterocytes which, in the ileum, can be identified by the presence of the large supranuclear vacuole (42). SI and ASS, therefore, form a very useful set of markers to follow enterocytes maturation. In aggregate, the findings indicate that the maturation of the enterocytes in the F/A-2 mice only distinguishes itself from that in wild-type mice by a slightly protracted time course.

Defective formation of Peyer's patches in homozygous F/A-2 mice

Arginine has been implicated as an important factor in lymphocyte proliferation and function (21). This prompted us to investigate the development of the immune system. At 3 weeks, the weight of spleen and thymus of homozygous F/A-2 mice is less than one fourth of that of wild-type animals of the same age (Table 3). Compared to wild-type pups of the same body weight (ND8), splenic weight is still decreased to 40%, whereas thymic weight is not significantly different.

<table>
<thead>
<tr>
<th></th>
<th>WT (ND21)</th>
<th>F/A-2</th>
<th>WT (ND8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bodyweight</td>
<td>13.5 ± 1.0</td>
<td>4.7 ± 0.3</td>
<td>4.8 ± 0.2</td>
</tr>
<tr>
<td>Splenic weight</td>
<td>67 ± 10(^a) (0.5)</td>
<td>15 ± 9(^ab) (0.3)</td>
<td>38 ± 1(^b) (0.8)</td>
</tr>
<tr>
<td>Thymic weight</td>
<td>79 ± 7(^c) (0.6)</td>
<td>17 ± 4(^c) (0.4)</td>
<td>24 ± 1(^c) (0.5)</td>
</tr>
</tbody>
</table>

Table 3. Splenic and thymic weights in wild-type and homozygous F/A-2 mice. Splenic and thymic weights of F/A-2\(^{-/-}\) mice are significantly lower than that of wild-type mice at ND21 (a,c; \(p<0.01\)). Splenic, but not thymic weight of F/A-2\(^{-/-}\) mice is also significantly reduced when compared to a weight-matched
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wild-type animal (c; p<0.01). %: organ weight as a percentage of body weight. Values are means ± SEM (n=12 for both groups).

Inspection of the intestines revealed that the development of Peyer's patches (PPs), the first macroscopically visible, morphological hallmark of the GALT, is also affected by arginine depletion. In the third postnatal week, 4-7 PPs are always seen protruding from the serosa of the small intestine of wild-type, homozygous F/A-1 and hemizygous F/A-2 mice (Fig. 7A). However, in a total of 23 small intestines of 18-days old homozygous F/A-2 animals, only 8 rudimentary PPs were identified macroscopically (Fig. 7B), instead of the more than 92 expected (p < 0.001). These remaining PPs were much smaller in size compared to the ones observed in wild-type small intestines (Fig. 6A',B').

Fig 6. Enterocyte maturation in wild-type and F/A-2+/− mice. Panel A: Western blots of jejunal tissue of wild-type and F/A-2+/− mice at ND14, 18, 20 and 24, probed with polyclonal antibodies against ASS, SI, A-I, and A-II.
Fig. 6, panel B: Expression of ASS and SI along the crypt-villus axis of the jejunum and ileum of wild-type (wt) and F/A-2"/" mice (+/+ ) at ND14, 16, 18, and 20. All sections were counterstained with haematoxylin.
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Fig. 6, panel B, continued.
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In homozygous F/A-2 pups that were supplemented with arginine, a normal number of normally sized PPs was found (Fig. 7C). No such effect was seen after treatment with saline or creatine. Normal development of PPs is, therefore, critically dependent on arginine availability. To establish whether or not PP organogenesis is disturbed, we performed whole mount immunostaining of the small intestine of 6-day old pups for the presence of VCAM-1, the first marker of PP development (43). This endothelial receptor for the integrin α4 is essential for lymphocyte homing to the PP. The number and size of VCAM-1-positive cell clusters (4-7) in small intestines of homozygous F/A-2 mice is indistinguishable from wild-type animals, demonstrating the presence of intact PP anlagen (Fig. 7D,E). In homozygous F/A-2 mice, PPs become macroscopically identifiable at 6-7 weeks after birth. These data show that development of PPs in F/A-2 mice is temporarily suspended, but not abolished. The development of the mesenteric and peripheral lymph nodes is undisturbed in homozygous F/A-2 mice. In order to reveal a possible mechanism for the effect of arginine on PP development, we investigated mice carrying null mutations of the three isoforms of the NOS gene (44-46) or the *spf-ash* mutation in the OTC gene. At ND18, the normal number and size of PPs was found in *spf-ash-* mice, as well as in nNOS-, iNOS-, and eNOS-deficient mice. This observation shows that the hampered development of PPs is not due to a deficient citrulline or nitric oxide production by any of these enzymes. In addition, the normal number and size of PPs in *spf-ash* mutants suggests that the failing development of PPs in F/A-2 homozygotes is not due to runting. In aggregate, the data suggest that the defective
The development of PPs in F/A-2 mice is caused by arginine deficiency rather than by deficiency or aberrant levels of one of its metabolic products.

**Fig. 7. Peyer's patch development in wild-type and F/A-2 transgenic mice.** Panels A and B: the small intestine of a wild-type (A) and a F/A-2/++ (B) at ND18, with arrows pointing at PPs. PPs were not macroscopically identifiable in F/A-2/++ mice. Panel C: arginine injections normalize the appearance of PPs in F/A-2/++ mice (arrows). Panels D and E: whole-mount immunohistochemical staining of the small intestine of a ND6 wild-type (D) and a F/A-2/+/− (E) animal for the presence of VCAM-1-positive PP anlagen (arrows). Note the presence of Peyer's patch anlagen in both genotypes. Panels A' and B': Haematoxylin and azophloxin-stained sections of a typical PP in a wild-type (A') and an F/A-2/++ (B') mouse. Bars: 0.6 mm (panels A-E); 3 mm (panels D, E), 75 μM (panels A', B').
Discussion

Requirements for the development of the F/A phenotype

Phenotypic characteristics of transgenic mice that express arginase in their enterocytes include a temporary retardation of body and fur growth and of the development of the immune system, including PPs. As demonstrated by the normal arginine concentration and phenotypic appearance of lines F/A-3 and -6, expression of the transgene in all enterocytes is necessary to elicit the characteristic phenotype. As demonstrated by the differences between hemi- and homozygous F/A-1 and -2 pups, the severity of the phenotype depends on the level of expression of transgenic A-I in the enterocytes, that is, the degree to which cytosolic arginase interferes with arginine metabolism. This assessment is underscored by the transient nature of the F/A-1 and -2 phenotype, which ameliorates after the arginine-synthesis in the small intestine stops (15). However, the results of the arginine injections show that the A-I overexpressing enterocytes also function as an effective arginine drain. Since F/A-2 adult males have a lower circulating concentration of arginine than females, they are apparently more sensitive to this effect than females. At present, we do not know whether the F/A phenotype will also develop if transgenic arginase is expressed in another tissue than the gut. If not, it would imply that the suckling gut requires a local source of arginine that is insufficiently met by the supply via the milk. We are presently exploring this issue.

The graded phenotype of the F/A transgenics reveals a relation between circulating arginine concentration and retardation of growth and development. The transient sparse-fur phenotype of F/A mice is also seen in OTC-deficient spf-ash mice. At 3 weeks, plasma and intestinal arginine concentration in homozygous spf-ash mice are approximately 120 µM (47, 48). Combining these data with our own, we conclude that the sparse-fur trait develops when plasma arginine in the suckling period drops below 120 µM. Hampered muscle and immune development is only seen in homozygous F/A-2 mice and, therefore, develops when plasma arginine concentration drops below 80 µM. The lack of a linear relation between intestinal arginase levels and plasma arginine concentration in the respective transgenic lines may be due to induction of arginine biosynthesis elsewhere, e.g. in the kidney (41).
Given the high $K_M$ of A-I, being in the millimolar range, such an extra-intestinal biosynthetic capacity may be sufficient to blunt the effect of increasing levels of arginase in the enterocytes.

The lack of normal body growth in homozygous F/A-2 mice appears to reflect impaired muscle development rather than starvation. In fact, the normal concentration of serum IgG, which is actively taken up from the milk (49) by specific neonatal Fc-receptors on the intestinal epithelium (37), shows that milk intake and absorption in F/A-2 mice is normal. The finding that the plasma and tissue concentration of all amino acids except arginine and ornithine are normal, and that the impaired growth of fur, muscle and PP development responds to arginine injections, further underscores the hypothesis that the observed developmental disorders solely result from an arginine deficiency.

**Causes of the development of the F/A phenotype**

Arginine is a precursor for the synthesis of proteins, creatine, agmatine, NO and polyamines and an intermediate in the detoxification of ammonia. We therefore evaluated these pathways for a possible cause of the F/A phenotype. Since the concentration of NH$_3$ and polyamines in F/A-2 mice is normal, these factors can be excluded. General protein synthesis is probably also not affected in our transgenic mice, as the $K_M$ of arginyl-tRNA synthetase for arginine is approx. 20 μM (50), i.e. well below the observed tissue arginine concentration in the F/A-2 transgenics. However, incomplete charging of arginyl-tRNA may affect proteins with a very high arginine content, especially if the arginines are adjacent (51). An example is trichohyalin, a major intermediate filament-associated protein in the inner root sheath and medulla of hair follicles with 21.5% of its amino acids consisting of arginines, including 70 diplets and 14 tripletts (52). Unexpectedly, no decrease in trichohyalin content was found in tissue sections and Western blots of F/A-2 skin. Since the steady state concentration of this extremely arginine-rich protein is apparently not affected, we conclude that arginine deficiency does not exert its effects on fur development and, hence, on neonatal growth at the translational level.
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Histologically, the muscle fibers of F/A-2 mice are 2-3 fold smaller than those of control animals and suggest delayed development. Although a positive effect of creatine supplementation on muscle mass is disputed, we tested it, since it was shown that metabolic deficiency of creatine can be corrected by substitution (53). From the absence of an effect, we conclude that the F/A phenotype does not result from a creatine deficiency. We therefore do not know at present how arginine regulates normal muscle growth. However, it is noteworthy that the muscular phenotype that develops as a result of α7-integrin deficiency, also becomes manifest soon after birth (54).

The decreased spleen weight and the tardy development of PPs indicate that the selective decrease in circulating and tissue arginine concentration retards the development of the immune system. In accordance with the relatively normal thymus-to-body weight ratio, our preliminary data indicate that arginine deficiency primarily affects B-lymphocyte development. To our knowledge, a direct stimulatory effect of a common nutrient such as arginine on immune development in vivo has not yet been shown, although the therapeutic potential of arginine as an immuno-nutrient in enteral and parenteral nutrition in a clinical setting was already claimed 20 years ago (55, 56). Because nontoxic concentrations of nitric oxide are thought to enhance Th2-dependent immune responses (57), PP development was investigated in nNOS-, iNOS-, or eNOS-deficient mice. In these mice, normal PPs were found, suggesting that the effect of arginine deficiency is not exerted via any of these enzymes separately. Unfortunately, mice deficient in all three NOS isoforms are not available to conclusively prove or disprove NO involvement.

In aggregate, our findings indicate that the F/A phenotype develops as a result of a deficiency of arginine itself, rather than with a deficiency of products of arginine metabolism. Clearly, the identification of the pathway that is primarily affected by arginine deficiency in the suckling period will be the next target of research. In this respect, the biology of PP development appears particularly promising, as homozygous F/A-2 mice share the combination of normal organogenesis, but disturbed maturation of PPs, in conjunction with normal lymph
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node development, with TNFα- and TNFR-1-deficient mice (58, 59). Our preliminary observation that F/A-2 mice have a drastically diminished number of mature B-cells in spleen and intestine, may represent another lead, as development of PPs, follicle-associated epithelium and M-cells was also found to be impaired in mice that lacked B-cells (60).

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